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THE UNIVERSITY OF ALBERTA

SEQUENTIAL CHANGES IN CANINE PULMONARY EPITHELIAL PERMEABILITY AND ENDOTHELIAL CELL METABOLIC FUNCTION AFTER NO_2

BY

DAVE J. WILLIAMS

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

EXPERIMENTAL RESEARCH

DEPARTMENT OF MEDICINE

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FALL 1988

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled SEQUENTIAL CHANGES IN CANINE PULMONARY EPITHELIAL PERMEABILITY AND ENDOTHELIAL CELL METABOLIC FUNCTION AFTER NO_2, submitted by Dave J. Williams, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN EXPERIMENTAL RESEARCH.

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This text is dedicated to

MARCELLE

my wife, my constant source of support and encouragement, and to

RODDY and UNA

my parents,

for their wisdom and love.

ABSTRACT

Toxic inhalation of Nitrogen Dioxide (NO_2), an oxidant gas is a practical problem due to occasional accidental occupational exposure. This gas produces a diffuse alveolar and terminal bronchiolar injury when inhaled in sufficient concentration. It is also an experimental tool which can be useful in the study of repair mechanisms. Study of the effects of NO_2 on the alveolar-capillary membrane may thus contribute both practical and theoretical benefits to inhalation toxicology.

Some of the methods that have been used to evaluate functional properties of the pulmonary epithelium in vivo include a) lung clearance of inhaled radiolabeled solutes and b) measurement of albumin in the epithelial lining fluid using bronchoalveolar lavage. With respect to the pulmonary endothelium, the removal of radiolabeled metabolic substrates from the pulmonary microcirculation has been promulgated as an advantageous method.

An attempt was therefore made to apply these techniques to evaluate simultaneously, pulmonary epithelial permeability and endothelial metabolic function after NO_2 inhalation. The first phase of this study involved thirteen mongrel dogs which initially breathed compressed air for 1 hour and then 200 ppm or 400 ppm NO_2 for 1 hour 2 days later. Changes in pulmonary epithelial permeability using a radioaerosol technique, functional residual capacity (FRC), static lung compliance (CL), and the removal of radiolabeled serotonin (^{14}C-5HT) and prostaglandin E_1(^{3}H-PGE_1) from the pulmonary circulation were evaluated at 1 hour and on day 2, 7 and 14 post NO_2 exposure. These findings were also correlated with morphological observations. The second phase involved the measurement of bronchoalveolar lavage albumin concentration in a second group of dogs that had an inhalational history similar to the first group of animals.

The pulmonary epithelial membrane was shown to be leaky. Despite the alteration in epithelial permeability, FRC decreased only slightly and static compliance was not altered. Microscopy showed focal and patchy pulmonary edema. No abnormalities in the removal of serotonin and prostaglandin E_1 were noted. All abnormalities returned to normal with time. This study therefore demonstrated that the barrier function of the epithelium is an early index of injury in NO_2 inhalation and that repair processes of the lung after NO_2 exposure are rapid.

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TABLE OF CONTENTS **Page**

ABSTRACT v

1. LITERATURE REVIEW
   Introduction 1 - 2

2. THE LUNGS AND PULMONARY VASCULAR SYSTEM
   a) Overview 3 - 5
   b) Alveolar capillary membrane stucture 5 - 9
   c) Fluid and solute exchange through the alveolar capillary membrane 9 - 11
   d) Effect of edema on pulmonary blood flow 12 - 13

3. NITROGEN DIOXIDE-INDUCED LUNG INJURY
   a) Morphological characteristics of NO_2 lung Injury 14 - 17
   b) Mechanisms of epithelial injury 17 - 19
   c) Factors modifying oxidant injury 19 - 20
   d) Lung mechanics of NO_2 lung injury 20 - 21

4. MEASUREMENT OF ALVEOLAR CAPILLARY MEMBRANE PERMEABILITY
   a) Indicator dilution techniques 22 - 23
   b) Fluid accumulation 23
   c) Lung lymph 23 - 24
   d) Protein turnover 24 - 25
   e) Radioaerosol clearance 25 - 28
   f) Factors influencing pulmonary clearance rate 28
   g) Animal species 29
   h) Site of aerosol deposition 29
   i) Molecular weight of solute 30
   j) Lung volume and pressure 30 - 31
   k) Surface lining of the respiratory tract 31

5. CONDITIONS ASSOCIATED WITH ALTERED RADIOAEROSOL CLEARANCE
   a) Acute lung injury 32

b) Inhaled gases 32-33

6. BRONCHOALVEOLAR LAVAGE AND ITS APPLICATION TO INHALATIONAL LUNG INJURY 34 - 37

7. PULMONARY MICROCIRCULATORY METABOLIC FUNCTION 38 - 40

8. EXPERIMENTAL
a) Introduction 41 - 43
b) Methods 43 - 45
c) Radioaerosol scanning 46 - 48
d) Bronchoalveolar lavage 48
e) Static pulmonary compliance 48 - 49
f) Functional residual capacity 49
g) Metabolic function of the pulmonary microcirculation 50 - 51
h) Morphological studies 51
i) Statistical analysis 51

10. RESULTS
a) Pulmonary physiological status 52
b) Radioaerosol scans 52 - 58
c) Bronchoalveolar lavage fluid 58
d) Hemodynamic parameters 58 - 62
e) Endothelial function 62
f) Histological Studies 62 - 67

DISCUSSION 68 - 79

CONCLUSIONS 80

REFERENCES 81 - 103

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1.	Different cell types in the parenchyma of normal adult human lungs.	6
2.	Factors influencing BAL recovery of soluble and cellular components.	35
3.	Experimental procedures performed in 13 animals.	44
4.	Ventilation and gas exchange parameters in dogs exposed to 200 ppm NO_2.	53
5.	Ventilation and gas exchange parameters in dogs exposed to 400 ppm NO_2.	54
6.	Sequential FRC responses to NO_2.	55
7.	Static compliance responses to NO_2.	56
8.	BAL Albumin and fluid volumes after air and 200 ppm NO_2.	60
9.	BAL cell count (total cells and percent differential post-air and post nitrogen-dioxide exposure.	61

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
I	Anatomic subdivisions of the terminal respiratory unit.	4
II	The alveolar capillary barrier and postulated pathways for solute translocation.	27
III	Experimental protocols used in the different groups of animals.	45
IV	Experimental circuit for radioaerosol scanning.	47
V	Pulmonary clearance of ^{99m}Tc-DTPA and $^{99m}TcO_4^-$ after 200 ppm NO_2.	57
VI	Pulmonary clearance of ^{99m}Tc-DTPA and $^{99m}TcO_4^-$ after 400 ppm NO_2.	59
VII	Sequential measurements of cardiac output and mean transit time after NO_2 exposure.	63
VIII	Time concentration and per cent removal curves following injection of ^{3}H-PGE_1 and ^{14}C-5HT into the right atrium of the one dog after compressed air.	64
IX	Sequential changes of removal of ^{3}H-PGE_1 and ^{14}C-5HT after NO_2 exposure.	65

LIST OF PHOTOGRAPHIC PLATES

FIGURE	DESCRIPTION	PAGE
1A	Light microscopy of dog lung post compressed air.	66
1B	Light microscopy of dog lung 1 hour post NO_2 exposure.	66
2A	Light microscopy of canine lung 2 days post NO_2 exposure.	67
2B	Light microscopy of canine lung 7 days post NO_2 exposure.	67

LIST OF ABBREVIATIONS

1.	A	- Cross sectional area
2.	AMAD	- Activity Mean Aerodynamic Diameter
3.	ARDS	- Adult Respiratory Distress Syndrome
4.	BAL	- Bronchoalveolar Lavage
5.	BTPS	- Body Temperature Ambient Pressure saturated with water vapor
6.	BCG	- Bromocresol green
7.	CL	- Static lung compliance
8.	cm	- centimeter
9.	CO_2	- Carbon dioxide
10.	CPAP	- Continuous Positive Airway Pressure
11.	CPM	- Counts per minute
12.	Cr-EDTA	- Chromium-labeled ethylenediaminetetraacetate
13.	Ctrl 1,2,3	- Control - (Pre and Post Compressed Air measurements)
14.	D	- Diffusion constant of a gas
15.	ELF	- Epithelial Lining Fluid
16.	FiO_2	- Inspired fraction of oxygen
17.	FRC	- Functional Residual Capacity
18.	H_20	- Water
19.	5HIAA	- 5-Hydroxyindoleacetic acid
20.	5HT	- Serotonin
21.	ICG	- Cardiogreen
22.	K	- Potassium
23.	Kf	- Filtration coefficient
24.	L	- Length
25.	LDH	- Lactate Dehydrogenase
26.	L/min	- Litres per minute
27.	MBq	- Megabecquerel
28.	MCi	- Millicurie
29.	µCi	- microcurie
30.	µL	- microlitre
31.	ml	- millilitre
32.	µmol	- micromol
33.	M.W.	- Molecular weight

34. Na - Sodium
35. nm - nanometer
36. NO - Nitric oxide
37. NO_2 - Nitrogen dioxide
38. N_2O_4 - Dinitrogen tetroxide
39. O_2 - Oxygen
40. P_{alv} - Alveolar fluid hydrostatic pressure
41. $PaCO_2$ - Arterial CO_2 tension (mmHg)
42. P_{ao} - airway pressure
43. PaO_2 - Arterial O_2 tension (mmHg)
44. PEEP - Positive end-expiratory pressure
45. PGE - Prostaglandin E
46. Ph - Logarithm of hydrogen ion concentration
47. PMNs - Polymorphonuclear neutrophils
48. P_{mv} - Microvascular or capillary hydrostatic pressure
49. P_{pl} - Pleural pressure
50. P_{pmv} - Perimicrovascular interstitial hydrostatic pressure
51. psi - pounds per square inch
52. PV - Pressure volume
53. P_1, P_2 - Partial pressures of gas
54. $\dot{Q}f$ - Net liquid flow
55. r -radius
56. ROI - Region of interest
57. SH - Sulfhydryl
58. SO_2 - Sulphur dioxide
59. T - membrane thickness
60. TB - terminal bronchiole
61. ^{99m}Tc-DTPA - technetium labeled diethylenetriamine pentaacetate
62. $^{99m}TcO_2$ - Hydrolyzed-reduced ^{99m}Tc
63. $^{99m}TcO_4^-$ - Sodium Pertechnetate
64. ^{99m}S-colloid - Technetium-labeled sulphur colloid
65. TLC - Total lung capacity
66. $T_{½}$ - Half life
67. $\dot{V}gas$ - Gas flow measured in units of volume time

68. $\dot{V}max$ - Maximum uptake rate

69. π alv - Alveolar fluid colloid oncotic pressure measured in mmHg

70. πpmv - Interstitial colloid oncotic pressure

71. σ - colloid reflection coefficient - measured in ml $mmHg^{-1}$ $hour^{-1}$

1. LITERATURE REVIEW

Introduction

Nitrogen dioxide (NO_2) is an environmental pollutant arising from emissions from automotive and other combustion engines as well as from coal burning plants (Guidotti 1978). In addition, a risk of acute exposure to NO_2 is associated with several work places, including agriculture, military, welding, aerospace, firefighting and the chemical industry (Shy 1973, Yockey et al 1980). Considerable evidence suggests that NO_2 exposure causes various biochemical, physiologic and metabolic changes in mammalian lungs (Mustafa and Tierney 1978) and see Guidotti 1978 for review.

The earliest recorded toxic inhalation of NO_2 occurred when a French merchant and his dog died after breathing concentrated nitric acid fumes from broken containers in 1804 (Ramirez 1974). The industrialization of Europe led to further cases in the chemical industry, mostly in Germany in the late nineteenth century (Wood 1912). About the same time as the chemical industry was developing in Europe, coal mining was changing its production techniques; "shot-firing" the exposed coal seam with small charges underground generated toxic quantities of NO_2 during misfires (Kennedy 1972).

Exposure in agriculture takes the form of silo fillers' disease. The fixed nitrates in certain crops especially corn, may be much increased under conditions of drought, premature harvest, or bright sunlight. In the warm, moist silo, organic acid products of fermentation may release clouds of NO_2 and dinitrogen tetroxide (N_2O_4). The unwary workers who enter the silo within a week of ensilage may inhale the gases at exceedingly high concentrations (Morrisey et al 1975). Although the clinical features of this syndrome were recognized as early as 1914 they were incorrectly attributed to asphyxiation from accumulated carbon dioxide (CO_2) (Hayhurst and Scott 1914). Occasional accidental exposures result from burning plastics, shoe polish (Morrisey et al 1975), and nitrocellulose. The 1929 Cleveland clinic disaster which took the lives of many patients and physicians resulted from the liberation of enormous clouds of NO_2 from burning nitrocellulose X ray film (Gregory et al 1969).

These toxic effects of NO_2 are of considerable practical and theoretical interest. Inhaled toxic gases induce their pathogenetic effects on the organism by producing abnormalities in pulmonary gas exchange or the mechanical characteristics of the lung which together or separately can cause symptoms of ventilatory discomfort, hypoxemia, hypercapnia, or alterations in acid-base balance. These alterations in physiologic function, however, are often the consequences of a change in structure of the lung from many forms of tissue injury. These include loss and distortion of alveoli, interposition of new tissue growth in the

alveolar-capillary membrane, changes in blood vessel and airway caliber as well as interference with the fine mechanical relationships between the distensibility and collapsibility of the lung parenchyma and the muscle function of the chest wall and diaphragm. These changes in tissue structure however, must have their beginnings as less easily detected alterations at the ultrastructure level of lung morphology. Therefore, the beginning of lung injury may take place as a disturbance in molecular structure of the central architectural components of lung tissue or as interruptions of metabolic functions at the cellular level.

Over the past few decades morphologic study of NO_2 inhalation of the lung with the assistance of ultrastructure visualization and cell tracer technic have defined these architectural consequences of NO_2-induced lung injury. They have ranged from destruction of lung parenchyma, as in emphysema, to hyperplasia of cellular elements of the lung.

In recent years, as a result of new aproaches to the investigation of lung structure and function at the level of ultrastructure and specific cell metabolic processes, it has been possible to recognize the occurrence of injury to lung structure at its earliest phases. Many different techniques have been used to detect clinically and experimentally produced inhalational lung injury. These include roentgenography, computerized axial tomography, measurements of both pulmonary mechanical function and gas exchange, indicator dilution techniques, bronchoalveolar lavage and radioaerosol clearance.

More recently, clearance of technetium-labeled diethylenetriaminepentaacetate (^{99m}Tc-DTPA) has been used to measure permeability of the alveolar epithelium in a variety of situations including chronic interstitial lung diseases (Rinderknecht et al 1980), patients with adult respiratory distress syndrome (ARDS)(Mason et al 1985) and hyperoxia (Griffith et al 1986). In these conditions clearance of the radioaerosol was increased. Other workers have used bronchoalveolar lavage (BAL) to examine changes in the permeability of the alveolar-capillary membrane after exposure to oxygen (Davis et al 1983). Increased amounts of albumin and transferrin have been found in BAL fluid suggesting an increase in permeability of the blood-air barrier in response to hyperoxic exposure.

With respect to the capillary endothelium, in models of inhalational lung injury, the pharmacokinetic function of lung as expressed towards substrates in the pulmonary circulation has been shown to be altered by inhaled gases (Block and Fisher 1977, Dobuler 1982, Chaudyri and Dutta 1980). The metabolism of both serotonin and prostaglandin E_1 have been proposed as test substrates for detecting early lung inhalational injury.

To determine the relative sensitivity of different methods in measuring sequential changes in lung injury, permeability of the alveolar capillary barrier (^{99m}Tc-DTPA and $^{99m}Tc0_4^-$ clearance, BAL albumin concentration), endothelial metabolic function (^{14}C-5HT, ^{3}H-PGE_1, metabolism), lung mechanics and edema were examined using the dog.

2. THE LUNGS AND PULMONARY VASCULAR SYSTEM

Overview

The function of the bronchial tree is to conduct air to the alveolar surface where gas transfer takes place between respired air and gas dissolved in the blood of the alveolar capillaries. The greater part of the length and the smaller part of the volume of the respiratory tract are concerned only with the conduction of air; this part comprises the trachea and bronchi (whose walls contain cartilage) and non-alveolated bronchioles (no cartilage). The remainder of the respiratory tract, comprising the large bulk of the lungs is concerned with both conduction and gas exchange; the terminal unit (the alveolus) being the only structure whose function is uniquely that of gas exchange. Thus the airway system can be subdivided into three zones each with different structural and functional characteristics.

The conductive zone includes the trachea, right and left main bronchi, the lobar bronchi, segmental bronchi and finally, the terminal bronchioles. There is minimal diffusion of gas through the well-developed wall of these latter structures. The conducting airways also include the portion between the nares and trachea. The volume of this conductive zone also called 'anatomical dead space' has a volume of approximately 150 millilitres (ml) in humans.

The transitory zone carries out both conductive and respiratory functions. It consists of the respiratory bronchioles, alveolar ducts and alveolar sacs, all of which conduct air to the most peripheral alveoli. In addition, alveoli that arise from their walls are the site of gas exchange, This zone and the respiratory zone constitute the parenchyma, the spongy respiratory portion of the lung (Figure I).

The respiratory zone consists of the alveoli whose sole function is the exchange of gases between air and blood. The number of alveoli may number as many as 300 million. The respiratory zone makes up most of the lung; its volume being about 3000 ml in humans. The inspired air moves through the conducting zone with minimal resistance to the respiratory zone. This movement is by bulk flow. Beyond the terminal bronchioles, the forward velocity of air is quite small as a result of the enormous increase in cross-sectional area occuring at the level of the alveolar ducts. In the respiratory zone gas is transferred across the blood-gas barrier. This process occurs by diffusion. Diffusion through tissues is described by Fick's Law. This states that the rate of transfer of a gas through a sheet of tissue is proportional to the tissue area and the difference in gas partial pressure between the two sides, and inversely proportional to the tissue thickness:

$$\dot{V}_{gas} \alpha \frac{A.D.(P_1 - P_2)}{\sqrt{T}\ mw}$$

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Figure I: A diagramatic representation of the anatomic subdivisions in a single plane through a terminal respiratory unit (ie. those structures distal to a terminal bronchiole, TB). Roman numerals indicate respiratory bronchioles; arabic numerals indicate alveolar ducts. (From Murray JF. The Normal Lung 2nd ed: Philadelphia, W.B. Saunders Company 1986; p. 44).

where V_{gas} is gas flow, A is the cross sectional area of the membrane and T is the membrane thickness and mw is the molecular weight of the gas. D is the diffusion constant of the gas, measured in ml x minute^{-1} and $(P_1 - P_2)$ is the difference in partial pressure of the gas across the membrane, measured in mmHg. The cross-sectional area of the blood-gas barrier in the lung is between 50 and 100 square meters, while the thickness is less than 0.5 micrometer (μm). Thus the dimensions of the barrier are ideal for diffusion in the respiratory zone.

The pulmonary circulation begins at the main pulmonary artery which receives the mixed venous blood pumped by the right ventricle. This artery then branches successively like the system of airways with the pulmonary artery accompanying the bronchi as far as the terminal bronchioles. Beyond that, they break up to supply the capillary bed which lies in the walls of the alveoli. The pulmonary capillaries form a dense network in the alveolar wall which makes an exceedingly efficient arrangement for gas exchange. The oxygenated blood is then collected from the capillary bed by the small pulmonary veins which run between the lobules and eventually unite to form four large veins (in man) which drain into the left atrium.

Alveolar Capillary Membrane Structure

The alveolar capillary membrane consists of 1) alveolar epithelium and its basement membrane, 2) capillary endothelium and its basement membrane, 3) contiguous tissue elements in the intercalated interstitial space and 4) surfactant lining. The alveolar macrophage is another cellular component of the terminal respiratory unit.

The proportions of the cell types that comprise the parenchyma of the normal adult human being have recently been analyzed (Crapo et al 1982) Table 1. The alveolar epithelium consists of a continuous layer of tissue made up of two principal cell types. Type I cells, or *squamous pneumocytes*, have broad, thin extensions 0.1 to 0.3 μm thick which contain mainly cytoplasmic ground substance and cover 93 percent of the alveolar surface. These highly differentiated cells do not divide and their large thin surface with sparse cytoplasm makes them extremely susceptible to injury from a variety of blood-born or inhaled/aspirated toxins. Type II cells or *granular pneumocytes* are more numerous than type I cells but owing to their cuboidal shape they occupy approximately 7 percent of the alveolar surface. The electron microscopic hallmarks of type II cells are their microvilli and lamellar osmiophilic inclusion bodies which are involved in surfactant storage and release. The type II cell is considered as the progenitor cell of the alveolar epithelium particularly after a variety of experimentally induced and clinical injuries of the lung parenchyma (Gail and Lenfant 1983).

Table 1: RESULTS OF STUDIES (mean +/- standard error) OF THE DIFFERENT CELL TYPES IN THE PARENCHYMA OF THE NORMAL ADULT HUMAN LUNGS*

Table 1 has been removed due to the unavailability of copyright permission.

* from Crapo J.D. et al: Cell number and cell characteristics of the normal human lung. *Am Rev Respir Dis* 1982; 125: 740 - 745.

The capillary endothelium is composed chiefly of the cytoplasmic extensions of the *endothelial cells* which by their contiguous arrangement form a thin vascular tube. Besides its important role in gas exchange, the capillary endothelium is considered to be the major site of liquid and solute exchange in the lungs. More recently it has been recognized as the locus of a variety of active biochemical processes; including the cellular uptake and degradation of biogenic amines, peptides and arachidonic acid metabolites (Gillis and Pitt 1982, Pitt et al 1981).

The alveolar epithelium and the capillary endothelium both rest on separate basement membranes. Because of the presence of structural differences that have important functional implications, the alveolar capillary membrane has been subdivided into two separate regions: 1) the "thin portion" of the septum or the air-blood barrier over which the two basement membranes appear to be fused and 2) the "thick portion" is found where the basement membranes are separated by an interstitial space containing fine elastic fibres, small bundles of collagen fibrils and an occasional fibroblast. The interstitial space is thus perforated by capillaries which weave back and forth in the interalveolar septum and are thought of as a communicating meshwork and not as a continuous sheet. This interstitial space of the interalveolar septum extends directly into the connective tissue sheath and the interstitial space surrounding airways and blood vessels. Diffusion of gases takes place mainly across the thin portion while liquid and solute exchange occurs mainly across the thick portion. The thickness of the thick side varies depending on lung size (Weibel 1972, Meban 1980). However, that of the thin side appears to be similar in all mammalian lungs owing to the similar thicknesses of the alveolar epithelium and capillary endothelium.

Surfactant is a complex mixture of surface active materials (i.e., substances capable of lowering surface tension), chiefly phospholipids and proteins present at the air liquid interface of the terminal respiratory units. It is produced by type II pneumocytes, stored in lamellar bodies of these cells and released into the overlying subphase of the alveolar epithelial membrane. It contributes to the recoil properties of the lung and plays an important role in stabilizing and supporting the lung parenchyma. On electron micrographs, surfactant appears as a finely granular contour with a mean thickness of approximately 4 nm. This surfactant lining layer is made up of 3 parts; a surface film composed of highly surface active phospholipids (mainly dipalmitoyl lecithin) and a deeper hypophase layer containing phospholipid carbohydrate and protein (Morgan 1971, Weibel 1968). This surfactant layer is of particular importance in inhalation lung injury and may also be important in alveolar-capillary membrane permeability changes in disease states.

The alveolar capillary membrane not only provides a large surface area for gas exchange but also acts as a barrier against the indiscriminate leakage of fluid from capillary to alveolus.

A number of adaptations exist which assist the membrane in its barrier functions. These include 1) a pulmonary capillary pressure which is below the oncotic pressure of serum proteins; 2) a surfactant system which reduces intraalveolar surface tension, and 3) an efficient lymphatic drainage system. On the other hand, a subatmospheric interstitial pressure promotes fluid leakage. Nevertheless, the intrinsic structure of the alveolar-capillary membrane itself does provide an effective barrier at the air-blood interface.

The alveolar epithelial cells, both type I and type II are joined to each other by tight junctions which appear to be much tighter than those in the capillary endothelium. Of the two components of the alveolar-capillary membrane, the alveolar epithelium is the limiting structure in terms of molecular flux (Chinard et al 1962, Taylor et al 1967, Taylor and Gaar 1970). Some investigators have used the concept of reflection coefficients and showed that pores in the alveolar epithelium (most likely representing tight junctions) were 0.8 - 1.0 nm in radius and those in the capillary endothelium 4 - 8 nm (Taylor and Gaar 1970).

Transmission electron microscopy of tight junctions has demonstrated that the plasma membranes from adjacent cells appears to fuse at several points along the length. Freeze fracturing techniques reveal that at the points of membrane fusion there are fibrils within the membranes, These fibrils form an irregular lace-like network (Schneeberger and Karnovsky 1976) and differ in various epithelia and in different parts of the same epithelium or endothelium (Bradley and Purcell 1982). Freeze fracture studies also show that the epithelial junctions are joined by a much more complex network than the endothelial junctions (Schneeberger and Karnovsky 1976). The latter are connected by only one or two strands which are discontinuous in some areas and may therefore leak small solutes.

There are at least three types of tight junctions in various parts of the pulmonary vasculature (Simionescu et al 1975). Of the three types of tight junctions seen, capillary arteriolar and venular; that of the arteriolar end appears to be the most complex and probably the tightest. Various workers have shown that these tight junctions are the major sites of macromolecular translocation across the endothelium (Michel et al 1977) and they can be widened by various disease processes (Boucher et al 1974, Rinderknecht et al 1980).

Cytochemical techniques have failed to determine the chemical nature of these tight junctions. However, they appear to behave like fixed-charged gels with negative sites. This is of some importance in solute and especially ion translocation. For example, in frog choroid plexus these negative sites make the junctions more permeable to cations than anions (Wright 1972). Furthermore, it has been shown that in fetal lamb lungs, cations traverse the epithelium more readily than do anions (Olver 1974).

Tight junctions are thus very important in determining alveolar-capillary membrane permeability. Various techniques have shown that clearance rates of solutes across the

alveolar-capillary membrane are inversely related to molecular weight. This hindrance of flux based on size criteria is compatible with a diffusive transfer of hydrophilic solutes. These intercellular junctions of the alveolar epithelium or zonulae occludentes thus form a structural barrier to the paracellular movement of large molecular size substances. The control of paracellular permeability is thus thought to be regulated through the structural configuration and continuity of the interconnecting strands composing the tight junctions (Claude and Goodenough 1973, Claude 1978). However others, (Muller 1980; Walker et al 1984) have suggested that the corner pore formed by the opposition of three or more adjacent cells may also be a route of permeation.

Part of the difference in the permeability of these two cellular layers may be due to the differing nature of the corresponding basal laminae. The epithelial basal lamina has a dense homogenous network of collagen fibres with fine 3 - 5 nm filaments running perpendicularly between basal lamina and the epithelial basal surface. The principal constituents of the epithelial basal lamina are type IV and AB_2 collagen, heparin sulfate proteoglycans and associated glycoproteins (eg. fibronectin and laminin) (Vaccaro and Brody 1981). In contrast, the endothelial basal lamina contains a less dense and more irregular array of heparin sulfate proteoglycans and as such display only one fifth the number of anionic sites as the epithelial basal lamina (Vaccaro and Brody 1981).

Fluid and Solute Exchange through the Alveolar Capillary Membrane

The physics of movement of fluid and solutes through the alveolar-capillary membrane is still a matter of intense debate. However, it is obviously clear that the amount of extravascular liquid in the lungs at a given moment depends upon the balance between the rate of formation of liquid and the rate of its removal. Net liquid flow (Q_f) across the endothelial and epithelial barrier of the alveolar-capillary membrane can be described by the Starling equations, (1) and (2) respectively:

$$\dot{Q}_f = Kf[(Pmv - Ppmv) - \sigma(\pi mv - \pi pmv)] \qquad (1)$$

$$\dot{Q}_f = Kf[(Ppmv - Palv) - \sigma(\pi pmv - \pi alv)] \qquad (2)$$

where Kf is the filtration coefficient of the solute in question measured in ml x $mmHg^{-1}$ x

hour $^{-1}$ which describes the permeability characteristics of the membrane through which exchange occurs; Pmv, Ppmv and Palv are the hydrostatic pressures in the microvascular, perimicrovascular interstitial spaces, and the alveolar fluid respectively; πmv, πpmv and πalv are the colloid osmotic (oncotic) pressures in the plasma, interstitial space and alveolar fluid; and σ is the colloid reflection coefficient, a term that defines the effectiveness of the membrane in preventing the flow of colloid compared with the flow of water.

The prinicipal site of liquid filtration under normal conditions is the pulmonary capillary endothelium but arterioles and venules are involved to some extent as well. Collectively, the vessels from which the fluid is filtered are called the microvasculature of the lungs. Normally there is a net outward filtration of liquid, which cannot be measured directly in humans but which is estimated to be 10 to 20 ml/hour in adults (Staub 1978). Normally a similar amount is removed from the lungs through the pulmonary lymphatic circulation to prevent the retention of excessive liquid. Pulmonary edema nearly always represents an enhancement of the processes favoring liquid filtration, but it can only occur when the rate of filtration exceeds the rate of removal.

The different rates of movement of small and large molecules through membranes are often explained by assuming that pores with varying dimensions exist; however, the anatomic counterparts of the postulated pore system in the alveolar-capillary membrane are not known and, at least in other organs, the pore theory has failed to account satisfactorily for the results of experimental studies of molecular transport (Renkin 1985). It is generally believed that liquid and solutes pass from the bloodstream into the interstitial space of the interalveolar septum through interendothelial junctions, which restrict the passsage of protein-sized, lipid-insoluble molecules. Thereafter, liquid is prevented from entering alveolar spaces by the presence of the relatively tighter interepithelial junctions.

Morphological evidence that the narrow portions of the endothelial tight junction can permit passage of solutes is based on studies (Schneeberger and Karnovsky 1971, Pietra et al 1969) which show that with the expansion of blood volume or increases of arterial pressure, horseradish peroxidase and hemoglobin can traverse the junctions. Morphologically, the area of the junctions represents less than 0.1% of the endothelial surface (Karnovsky 1970) and it has been estimated that only one-thirteenth of the junctional area is actually open (Perl 1971).

The transport of proteins and other macromolecules is poorly understood and highly controversial (Schneeberger 1983). Nevertheless, ultrastructural studies clearly show the entrance of macromolecules into endothelial pinocytotic vesicles (Palade et al 1979), but the contribution of the vesicular system to overall protein transport is uncertain. Recent evidence (Schneeberger 1983), indicates that plasma proteins interact with the glycoprotein layer on the

surface of endothelial cells to form another barrier that may be responsible for some of the molecular sieving properties of the endothelium. The alveolar epithelium is a tighter barrier to liquid and solute filtration than the microvascular endothelium, and it is more restrictive to protein movement as well.

Once filtered, liquid does not accumulate in the pericapillary interstitial space of the interalveolar septum. Instead, liquid flows into the interstitial spaces surrounding airways and blood vessels where the lymphatic capillaries are located and through which liquid is removed from the lungs. The continuity between the perimicrovascular and peribronchovascular interstitial spaces provides the necessary pathway for the movement of filtered liquid from its site of formation to its site of removal from the lungs through the pulmonary lymphatics. As pulmonary edema occurs, substantial volumes of liquid accumulate in the peribronchovascular spaces which can be viewed as a large reservoir that must first be filled before liquid overflows into the alveolar spaces. This peribronchovascular fluid accummulation called "peribronchial cuffing" is clearly seen on chest x-rays of humans in congestive heart failure.

Permeability in a biologic system denotes the ease with which substances cross membranes, including the pulmonary vascular endothelium and the alveolar epithelium. All molecules do not move across pulmonary capillaries with equal freedom; some are more selectively retained within the bloodstream than others. In general low molecular weight substances cross more readily than those with high molecular weights, but lipid solubility, electrical charge, and molecular orientation are also important. In the Starling equation the filtration coefficient describes the porosity or leakiness of the membrane for water. When the permeability of the endothelial membrane increases, the filtration coefficient increases and the reflection coefficient decreases; accordingly then, more liquid is filtered at any given balance of forces across the membrane. Larger molecules in the blood have freer access to the interstitial space when permeability is increased. Because the alveolar epithelium is normally much less permeable than the vascular endothelium, the liquid that constantly flows into the perimicrovascular interstitial space is prevented from continuing into the alveolar space. Of note, the filtration coefficient does not include the dimensions of the membrane through which filtration occurs. If the surface area doubles and other conditions are unchanged, filtration will also double. Because of the complexity of the system, measurement of the permeability characteristics of the barriers to liquid and solute movement in the lungs in health and disease has proved extremely difficult.

Effect of Edema on Pulmonary Blood Flow

In a classic study on the effect of noxious gases on the lung, Sir Joseph Barcroft (Said 1964) stated, "The pathology of gas poisoning has been full of surprises but never was I more surprised than when I discovered that it was possible for a goat to live with its arterial blood, fully or almost fully, oxygenated, but with its lung four times its ordinary weight." He went on to speculate in what must have been one of the earliest statements of the ventilation-perfusion concept, that this could only be so if blood flow to the edematous regions of the lung was markedly reduced. Although gas poisoning probably causes pulmonary edema by increasing capillary permeability, in high pressure pulmonary edema gas exchange is maintained until gross edema occurs, so that interference with gas exchange by edema is an abrupt terminal event (Muir et al 1972, Said et al 1964).

The quantity of blood flowing through a given region of the lung is determined by the resistance of the vessels in that region and the driving pressure causing blood to flow through them. Therefore, if pulmonary edema were to affect the blood flow received by a particular lung region, it must do so either by changing the resistance of the vessels in that region or by changing the pressure driving through them. Before trying to understand the possible mechanisms by which regional pulmonary blood flow is affected by pulmonary edema it is necessary to review flow of fluid through tubes. Flow through rigid tubes is governed by Poiseuille's equation:

$$\text{Flow} = \Delta P \times \pi/8 \times 1/\eta \times r^4/L$$

where ΔP is the pressure difference between two intramural points, $1/\eta$ is the factor relating to the viscosity of the fluid, and r^4/L is a geometric factor relating to the vessel radius (r) and its length (L). Of course this equation is not strictly applicable to distensible tubes such as blood vessels because an increase in driving pressure will increase the intraluminal pressure so that the vessel will dilate and lengthen. The vessel radius is of critical importance as small changes in the radius of the vessel will markedly change flow, because the effect is related to the fourth power of the radius. Length changes on the other hand will have a much smaller effect in the opposite direction so that the overall effect of increasing the transmural pressure of the vessel will be to decrease vascular resistance and increase flow.

The sequence of changes in the lung as pulmonary edema develops has been described in detail (Staub et al 1967). Briefly, the fluid appears to collect in the bronchovascular interstitial space, then in the alveolar wall and at the corners of the alveoli in the airspace, and finally rapidly fills the airspace, reducing the volume of the filled alveoli. Interestingly, the airspace filling is patchy; the alveoli either become completely full or remain empty of fluid.

Theoretically, pulmonary vascular resistance could be altered by pulmonary edema in several ways. First, the collection of fluid in the bronchovascular interstitial space could raise the interstitial pressure and decrease the transmural pressure of the extraalveolar pulmonary vessels so that their resistance increases. Second, if the widening of the alveolar walls indicates fluid collecting in the alveolar wall interstitial space, this could decrease the transmural pressure of the capillaries and increase their resistance. Third, when fluid collects in the alveolar space so that alveolar gas pressure is replaced by the liquid pressure of the edema fluid, this could compress capillaries and increase their resistance. Fourth, as alveolar edema also produces partial collapse of the fluid-filled alveoli, this reduction in lung volume could affect the resistance of the extra-alveolar vessels; with the reduced volume the perivascular interstitial pressure would become more positive so that the transmural pressure of the extra-alveolar vessels would decrease. It is also possible that several of these mechanisms might operate simultaneously, so that the local change in the pulmonary resistance in edema may be a result of a complex combination of mechanisms.

Taken together these results suggest that the mechanism responsible for redistributing blood flow in acute pulmonary edema is associated with alveolar filling and probably due to the alveolar wall component of airspace edema. That the blood flow is well maintained so long as the airspace is empty and is reduced sharply when the alveoli becomes edema-filled provides an efficient mechanism whereby blood flow can be reduced to areas of the lungs that can no longer be ventilated.

3. NITROGEN DIOXIDE-INDUCED LUNG INJURY

Morphological Characteristics of NO_2 Lung Injury

It is not surprising that the lung is vulnerable to pollutants or toxins in the air, given that an adult human inhales approximately 3000 kg or 3 tons of air per year. Inhaled NO_2 may react with the mucus lining the respiratory tract, but generally the underlying epithelial cells are readily affected and the mucociliary transport is decreased (Guidotti 1978, Dalhamn and Sjoholm 1963). The delicate appearing ciliated cells of the airways and the very thin type I cells of the alveoli occupy a huge surface area that is exposed to inhaled pollutants and these cells are readily injured. Furthermore, the lung has the largest endothelial surface area of any organ and endothelial cells are also susceptible to toxins. Some inhaled toxic agents affect not only the epithelial cells but also the interstitial and capillary endothelial cells. On the other hand, a highly reactive substance such as ozone in relatively low concentrations (less that 1 ppm) may primarily injure the epithelium, because it reacts with epithelial cells before reaching the underlying cells. Therefore, inhaled toxins have different effects, depending on their particulate or gaseous nature, their solubility, their reactivity and the ease with which they can be removed from the lung or denatured.

Extensive histological examinations of the pathology induced by various doses of NO_2 have been performed by many workers. Concentrations which have caused death from acute pulmonary edema in man have been poorly documented, but indirect evidence indicates they were in excess of 100 ppm. Human subjects exposed to silo gas containing up to several hundred parts per million of NO_2 have developed acute pulmonary edema and, if they survive, a diffuse alveolitis and bronchiolitis obliterans follow (Grayson 1956, Moran and Hellstrom 1958). Lowry and Schuman (1956) coined the term "silo filler's disease" to categorize such patients.

Acute exposure to NO_2 results in a sequence of damage, inflammatory response, proliferation and repair in the lung. Morphological damage to type I epithelial cells and ciliated bronchiolar cells is first seen after 4 hours of exposure to 17 ppm NO_2 (Stephens et al 1972). Damage to epithelium results in increased permeability as evidenced by transudation of serum protein (Denicola et al 1981, Selgrade et al 1981) and penetration of high molecular-weight tracers (Gordon et al 1983). Exposure to NO_2 is also followed by recruitment of inflammatory cells into the lung with increases in polymorphonuclear neutrophils (PMN) (Denicola et al 1981, Gardner et al 1969) and macrophages (Denicola et al 1981). Repair of lung epithelium

after NO_2 exposure involves proliferation of alveolar type II cells and nonciliated bronchiolar cells (Evans et al 1972). The magnitude and rate of onset and decline of the edema, inflammation, proliferation and repair vary substantially with the concentration of NO_2, the duration of exposure, and the animal species under study.

Exposure to relatively high concentrations of NO_2 produces two main pathologic stages: 1) exudative (edematous) and 2) proliferative phases. The exudative phase begins within hours and pathologic changes include interstitial and alveolar edema formation of hyaline membranes and destruction of alveolar type I epithelial cells and capillary endothelial cells. The proliferative phase begins 2 to 3 days later and is characterized by hyperplasia of alveolar type II cells and of interstitial cells. These changes of NO_2 toxicity are most severe in the proximal acini, ie. near the terminal bronchioles (Freeman et al 1969, Stephens et al 1972). Morphologic changes in animal lungs exposed to NO_2 suggest that young cells that replace injured cells may be at least partially responsible for the metabolic changes. In the alveoli, the most sensitive cells appear to be the type I epithelial cells and the endothelial cells. Once injured, the type I epithelial cells are rapidly replaced by type II cells which may then transform into young type I cells (Evans et al 1975). Presumably injured endothelial cells are also replaced by young cells. The stimulus to replication of the interstitial cells is not known.

Morphometric studies by electron microscopy of dogs exposed to 37 ppm of NO_2 by unilateral direct lung ventilation for 4 hours revealed several early changes primarily affecting the endothelium. These included loosening and disorganization of the interstitial structure, suggesting swelling and redundancy of the endothelial surface, and increased numbers of vesicles in the capillary endothelium (Guidotti 1980). Endothelial ballooning was also seen in rats exposed to 17 ppm for 8 hours (Stephens et al 1972). It has therefore been suggested that the earliest evidence of toxicity after NO_2 exposure appears to be the vascular endothelium. This is compatible with the concept that the endothelial cell is a highly, metabolically, active cell rather than as a passive vascular lining quiescent cell (Jaffe 1977). Relatively selective injury to the endothelium would explain many aspects of the inhalation toxiology of NO_2 by providing a mechanism for increased capillary permeability, release of vasoactive substances and perhaps injury to interstitial cell components (such as fibroblasts) by removing a protective shield (Thorgeirsson and Robertson 1978). However, (Stephens et al 1972) exposure of rats to 17 ppm of NO_2 for 8 hours revealed that injuries were primarily to the epithelial cells lining the terminal bronchiole and adjacent alveoli together with evidence of leakage of plasma into the alveolar space as denoted by the appearance of fibrin in the alveoli. Within 4 hours of exposure to 17 ppm of NO_2 swelling of type I epithelial cells was noted; the cells lining

the terminal bronchiole had begun to change and isolated foci of membrane destruction including sloughing and denuding of the epithelial basement membrane was demonstrated. This destructive phase of the early NO_2 lesion reached a maximum about 24 hours after the onset of exposure to 17 ppm of NO_2 and was followed by a period of adaptation and repair.

The reason for the multifocal nature of the NO_2-induced lung lesion is not well understood. It has been suggested (Gross et al) that it occurs because the ratio of the alveolar surface to the respiratory bronchiolar surface is so large and therefore more noxious particles are allowed to accumulate on the terminal bronchioles than in the alveoli. Others (Stephans et al 1972) postulate that the smallness of the terminal bronchiole, through which the air must pass compared to the size of the alveolar area is an important factor. Another factor is related to the solubility of the gas in tissue fluids. NO_2 has a low solubility and would be more likely to reach the periphery of the lung than eg. chlorine or sulphur dioxide which have high solubilities. Thus, some workers (Stephens et al 1972) have found that the cells most sensitive to NO_2 are the type I alveolar epithelial cells and the epithelium of the terminal bronchiole. These findings are contrary to the other reports which state that endothelium is destroyed (Dowell et al 1971).

Many studies have focused on the acute effects of NO_2 on respiratory epithelium. The effects have been shown to induce proliferation of clara cells (Evans et al 1972) and type II pneumocytes (Evans et al 1973) and cilia changes (Ranga et al 1981). One important reported effect was the ability of NO_2 to increase epithelial permeability (Case et al 1983, Gordon et al 1983). When hamsters were exposed to NO_2 for as brief a period as 6 hours there was a substantial disruption of tight junction fibrils in bronchioles and immediately surrounding alveoli, the fibrils were reduced in number and fragmented (Case et al 1983). When the lungs were exposed to NO_2 for 48 hours and allowed to recover for an equal amount of time tight junctions exhibited almost complete restoration of normal fibril number and organization (Case et al 1983). Focal fragmentation of fibrils was the only residual of the brief NO_2 (Case et al 1983). This physiological study also revealed that during NO_2 exposure 75% of bronchiolar tight junctions and 30% of alveolar tight junctions were porous to horseradish peroxidase instilled in sites via a trachea cannula (Case et al 1983). After 48 hours, recovery from the 48 hour NO_2 exposure showed complete restoration of barrier function to horse radish peroxidase (Gordon et al 1983). This data correlated well with the general appearance of tight junctions of freeze fracture replicas from adjacent specimens and specimens from similarly treated animals. Tight junctions between adjacent bronchiolar epithelial cells and between adjacent type I

pneumocytes were considered more sensitive to continuous NO_2 exposure (Gordon et al 1986). Junctions involving type II pneumocytes were also affected but apparently not to the same degree as adjacent type I pneumocytes. Disruption of tight junctions observed after NO_2 treatment indicates that during such exposure the bronchiolar and alveolar epithelium may not be capable of maintaining barrier function.

NO_2-induced alterations of barrier function throughout the airways and alveoli of the lung have been demonstrated for other agents such as cigarette smoke (Simani et al 1984) and ozone (Bhalla and Crocker 1984). It had been shown that the leakiness of the epithelium was due to disruption of tight junctional fibrils as revealed by freeze fracture technology (Case et al 1983). These data suggest that any oxidant gas may induce this type of intercellular membrane change. Such changes may be the result of ionic changes similar to those described for surfaces of type I pneumocytes (Gordon 1984) which may be induced by the interaction of the gas or its dissolved anionic form (NO_3) interacting with the glycoproteins or lipid constituents of the membranes.

Almost complete restoration of normal tight junctional ultrastructure after short term exposure (Case et al 1983) indicated that the bronchiolar and alveolar epithelium may be capable of restoring and maintaining normal barrier function after acute exposure. Therefore, acute NO_2 effects may temporarily interfere with the capacity of the bronchiolar and alveolar epithelium to maintain barrier function.

Mechanisms of Epithelial Injury

Nitrogen dioxide and its precursor, nitric oxide (NO) appear to be normal constituents of air. Nitrogen dioxide is the more stable form. Both of these gases have free radical potential because they contain unpaired electrons. The precise mechanism of NO_2 induced injury is unknown. However, one of the major theories involves free radical mediated peroxidative cleavage of membrane lipids (Guidotti 1978, Mustafa and Tierney 1978) which in mammalian lungs, has been reported to lead to the formation of the lipid hydroperoxides (Sagai et al 1984, Sevonian et al 1979). Free radicals are generally highly reactive and participate in physicochemical reactions such as hydrogen abstraction, radical addition, bond scission and annihilation reactions, that can oxidize unsaturated fatty acids in cell membranes, damage DNA and oxidize protein amino side chains (Pryor and Lightsey 1981, Thomas et al 1968, Proctor and Reynolds 1984). Although NO_2 reacts slowly with olefinic compounds by addition, it can

induce autoxidation of organic compounds at concentrations as low as 0.1 ppm (Chao and Jaffe1972). In addition to direct oxidation NO_2 can react with biologic systems by initiating free radical reactions and by promoting cis to trans isomerization which alters the geometry of unsaturated fatty acids (Mead 1976, Thomas et al 1968, Roehm et al 1971, Menzel 1976).

Various authors have viewed the mechanisms by which oxidizing gases cause cellular injury (Pryor 1982, Menzel 1984). NO_2 is an oxidant gas which can react with many biochemical moieties to form free radicals. Free radicals thus formed by these interactions may impair the structure and function of cellular components such as proteins, especially enzymes containing sulfhydryl groups (SH), nucleic acids and more importantly the cellular plasma membrane (Man and Hulbert 1986). The plasma membrane is the outer limiting structure of the cell; therefore, its permeability characteristics regulate the molecular species which enter and exit the cell and its organelles. This membrane is composed of lipoproteins, rich in polyenoic long chain fatty acids which are prone to undergo peroxidative decomposition under certain conditions.

Recently it has been demonstrated (Patel and Block 1986) that membrane fluidity was decreased and lipid peroxide formation increased in cultured endothelial cells after 5 ppm NO_2 exposure. This alteration in membrane fluidity has been shown to affect a variety of fundamental cellular functions including transmembrane transport enzyme action, growth characteristics and receptor-ligand interaction (Shinitzky 1984). In addition to decreasing plasma membrane fluidity, NO_2 exposure caused significant decreases in 5HT uptake and significant increases in Lactate dehydrogenase (LDH). The 5HT uptake and LDH release depend on the integrity of the plasma membrane and have been used before as indexes of plasma membrane injury in endothelial cells (Cummiskey et al 1981, Ody and Junod 1985, Ryan and Ryan 1977). These alterations in membrane-dependent properties and the fluidity alterations thus indicated that the plasma membrane is an important target in NO_2-induced cytotoxicity. Moreover, the results suggested that the altered fluidity was responsible for the decrease in the transmembrane transport of 5HT and the increase in LDH release.

Besides being involved in vital transmembrane transport processes, the plasma membrane provides a semipermeable barrier for the cell and participates in mitogenesis, cell cycling, differentiation, proliferation and transmembrane signal transduction (Shinitzky 1984, DeLaat et al 1984). Alterations in these fundamental cellular processes could have serious consequences for cell and organ function and viability. For example, the development of pulmonary edema in many mammalian species after NO_2 exposure may be caused by increased permeability secondary to NO_2-induced injury to the surface membrane of airway epithelial or capillary

endothelial cells (Guidotti 1978, 1980).

Factors Modifying NO_2 Lung Injury

Factors modifying the sensitivity of the lung to NO_2 injury include species, age, concentration of the gas and duration of exposure. Other factors include temperature, nutritional factors, humidity, airflow and concurrent lung injury by other agents. The determinants of oxidizing lung injury have been summarized (Man and Hulbert 1986). A major shortcoming of studies of NO_2 inhalation injury is incomplete morphologic and physiologic evaluation. It has therefore been suggested that morphologic investigations combine the classic techniques of light microscopy and transmission electron microscopy and scanning electron microscopy (Dungworth et al 1976). Furthermore it has been suggested that some physiological measures of the functional status of the epithelium be also simultaneously evaluated (Man and Hulbert 1986).

The structural changes induced by short and long term exposure to nitrogen dioxide have been extensively studied. There is substantial variation between species in susceptibility to NO_2 induced changes; the hamster, for example, is resistant to the emphysematous changes induced in mice very easily (Kleinerman and Cowdrey 1968, Kleinerman and Rynbrandt 1976). Swine are also resistant (Giddens et al 1970).

The age of the experimental animal has been demonstrated to modify the response to NO_2 inhalation (Cabral-Anderson et al 1977; Evans et al 1977). Studies in male rats age 1 to 25 months old showed that injury was more severe and the type II proliferative response was greater in the older rats but that there was a slower onset of repair in these older rats. This finding suggested that the age of the animal was an important factor in determining both the extent of tissue damage and the subsequent repair processes. It was also demonstrated that the type I alveolar cells were more sensitive to injury in aging than in young rats and that the ciliated and type I pneumocytes sustained injury at these sublethal exposures whereas the nonciliated bronchiolar (Clara), type II and capillary endothelial cells did not.

The influence of antioxidants such as vitamin E and selenium on oxidizing gas lung injury has been evaluated by several workers (McMillan and Boyd 1982, Elsayed et al 1983). It has been suggested that Vitamin E may be preferentially oxidized by oxidizing gas, thereby preventing the formation of lipid peroxide, or this antioxidant may react with free radicals produced by lipid peroxidation (Toppel 1982). However, a recent study on the influence of vitamin E and the age of animals (Stephens et al 1983) on lung morphology of pups concluded

that the age of the animal and not the vitamin E levels was the major determinant of the tissue responses.

The concentration of NO_2 delivered to the lower respiratory tract may be much lower than that inhaled; clearance of NO_2 in the upper respiratory tract is between 31 and 78% in rabbits and up to 90% in dogs (Dalhamn and Sjoholm 1963, Vaughan et al 1969). Early endothelial injury in endotracheally intubated beagles ventilated with 3 ppm NO_2 for 1 hour has been reported (Dowell et al 1971). A discrepancy exists with respect to dose when compared with other studies by Guidotti (1980). The studies by Dowell et al (1971) describe severe toxic effects at unusually low concentrations for brief durations of exposure, including pulmonary edema after 7 ppm for 1 hour and shock with circulatory collapse after 16 ppm for 1 hour. Their findings contrast with earlier studies (Carson et al 1962, Lewis et al 1969, and Riddick et al 1968). The latter workers exposed dogs to 53 ppm for up to 1 hour and to 25 ppm for 6 months in a chamber respectively without observing severe toxic effects. The results of Dowell et al (1971) are thus at variance with most other studies and may suggest exceptional susceptibility in the beagle strain used by Dowell et al compared to those used by other investigators (Lewis et al 1969, Guidotti 1980).

Lung Mechanics of NO_2 Lung Injury

In healthy and asthmatic subjects inhaling 4 ppm NO_2 no demonstrable pulmonary function responses were noted (Linn et al 1985). On the other hand guinea pigs inhaling 5 to 13 ppm of NO_2 for 1 hour had markedly increased airway resistance and respiratory frequency yet maintained a constant minute ventilation (Murphy et al 1964).

Several controlled clinical studies evaluating responses of healthy subjects to NO_2 are available. Although a recent report (Bylin et al 1985) noted an increase in airway resistance after a 20 minute exposure to 0.25 ppm and a decrease in resistance after exposure to 0.5 ppm NO_2, most NO_2 inhalation studies have shown no alteration in airway function in healthy human volunteers.With respect to asthmatics, no significant bronchoconstrictive effect was noted (Linn et al 1986), even with heavy exercise at concentrations approaching the maximum allowed in occupational exposures (Linn et al 1985). At the same time these results seem inconsistent with others' findings of bronchoconstriction or increased bronchial reactivity in asthmatics exposed to much lower (ambient-like) NO_2 concentrations (Bauer et al 1984, Roger et al 1985). These human data as expected however used only low-level NO_2 exposures.

Despite these conflicting reports a study of pulmonary ultrastructure and compliance in

dogs after short term exposure to NO_2 noted that only when atelectasis or intraalveolar edema occurred did static compliance become abnormal (Dowell et al 1971) . Moreover, only at 7 ppm NO_2 or above did compliance changes occur.

Very few studies have addressed the problem of the effects of high concentrations of NO_2 on lung volumes. It is assumed that if NO_2 causes pulmonary edema and alveolar debris that lung volumes would decrease and that compliance would decrease. Compliance has traditionally been employed for detecting the presence of excess fluid within tissues. Unfortunately this method has thus far failed to provide a reliable method for detecting the initial cellular dysfunctional stage of this pathological condition; for perceivable alterations in lung mechanics are present only in conditions which exhibit roentgenographic or auscultatory characteristics of late pulmonary edema.

The most appropriate pulmonary function parameter for assessing responses to NO_2 is uncertain. Measurements of airway resistance are most sensitive to changes in central pulmonary airways. NO_2 is a relatively insoluble gas that penetrates to the peripheral lung with its major site of injury at the terminal bronchiole and alveolus. Therefore, measurements of forced flow rates may be more appropriate for detecting changes in the peripheral airways, an impossibility however in anesthetized animals.

4. MEASUREMENT OF ALVEOLAR-CAPILLARY MEMBRANE PERMEABILITY

Indicator Dilution

Various techniques have been employed to assess the permability characteristics of the alveolar-capillary membrane. Chinard (1980) was one of the first to use the multiple indicator dilution technique. The principle of this method was that multiple indicators could be injected into the pulmonary circulation and the disappearance of a test indicator could be compared to an indicator known to be impermeant by means of several blood samples. The idea is that the differences between the indicator that does not escape at all from the vascular compartment and the indicator that does escape but is "barrier-limited" (that is limited in where it can go; primarily by hindrance at the capillary wall) is a measure of the rate of escape of the permeable substances from the microcirculation.

These initial studies had several limitatons. In vivo studies were limited to first pass measurements because of unknown volumes of distribution of the test indicators. Although corrections could be made, these required the labeling of the entire circulation which proved to be expensive. For substances with low permeabilities much longer time periods were required and these of course lead to further problems related to volume of distribution and excretion. Chinard also used this technique to assess movement of substances from alveolus to capillary (Chinard 1962). In this method indicators were placed in a small volume of fluid in the lung and flux was assessed by measuring the rising concentrations of the substances in the pulmonary circulation. Again, similar problems were present.

The problem related to in-vivo studies was largely overcome by in-vitro lung preparations. This technique when used in dogs, (Taylor et al 1964) showed that the alveolar membrane behaved like other cell membranes in that it was permeable to water and DNP, a fat soluble substance but much less permeable to Na^{24}(Rinderknecht et al 1980), K^{42}(Fromter 1972) and urea. These studies concluded that the alveolar epithelium was the limiting barrier of the alveolar capillary membrane.

Egan (1983), using a slightly modified technique for in vivo experiments isolated a segment of lung by wedging a catheter in the bronchus. The airspace distal to the catheter was collapsed and then filled with normal saline solution containing several radiolabeled hydrophilic solutes of known molecular sizes. An impermeant solute (eg. albumin or inulin) was also included in the cocktail. Measurement of the concentrations of the solutes in the alveolar liquid revealed that the impermeant solutes had parallel slopes and increasing concentrations with time because of the absorption of water, whereas the permeant solutes had decreasing

Other investigators (Theodore and coworkers 1975) looking at the flux of large polar solutes used the opposite approach. They instilled saline into a degassed portion of the left lower lobe and, following an intravenous injection of sucrose, inulin and dextran, measured increasing concentrations of these substances in alveolar fluid. They basically concluded that flux of these substances in the saline-filled dog lung model was significant and proposed that large pores must exist in the alveolar epithelium. All these studies are susceptible to artifacts introduced by filling the alveoli with fluid. These techniques have limited applications in clinical medicine. Although they help document the overall permeability characteristics of the membranes to specific substances they are invasive and cumbersome to be used in clinical situations. Other potential problems include dilution of instilled fluid by airspace lining fluid, fluid accumulation and reabsorption within the lungs.

Fluid Accumulation

One experimental approach to detecting increased permeability is to compare the rate of lung fluid accumulation resulting from increased vascular hydrostatic pressure with the rate resulting from some manipulation that is thought to increase vascular permeability (Grega et al 1979, Snell and Ramsey 1969). Methods for estimating lung water content include indicator dilution techniques (Chinard et al 1962, Goresky et al 1969), post mortem desiccation (Pearce et al 1965) and continuous weighing of isolated perfused lungs (Gaar et al 1967). Studies based solely on indicator dilution estimates of lung water content are difficult to interpret because of the baseline variability of the method and because the fraction of total lung water measured by this method is different under different circumstances (Pearce et al 1965).

All studies based on increased lung water content as a criterion for increased microvascular filtration are necessarily insensitive. In response to increased vascular pressure, filtration (as reflected in lung lymph flow) increased with moderate pressure but fluid did not accumulate until pressures were substantially elevated (Guyton and Lindsey 1959). Under some circumstances when vascular permeability is increased, microvascular filtration may increase ten-fold without an increase in lung water content (Brigham et al 1974). Thus pulmonary edema (ie. fluid accumulation) is only the tip of the iceberg.

Lung Lymph Flow

An alternative approach to assess endothelial flux is to measure lymph flow rates in chronic animal preparations (Staub 1978). Under steady state conditions lung lymph flow

represents the net volume of fluid leaving exchanging vessels and lymph protein concentration represents perimicrovascular protein concentration. Staub(1978) used a chronic sheep preparation in which the efferent duct of the caudal mediastimal lymph node was cannulated and the tail of the node resected to eliminate lymph not originating in the lung. Using this preparation Brigham and Associates (1976) have studied the sieving characteristics of the endothelial barrier by infusing dextrans of different sizes into sheep and observing their partitioning between blood and thoracic duct lymph. Wasserman et al (1979), using a similar technique in dogs found that the permeability of the endothelium was related to the molecular weight of tracer and that interestingly the endothelium of the dog was less permeable than that of sheep.

The problems with any lung lymph preparation are 1) estimation of hydrostatic pressure in exchanging vessels, 2) unknown perimicrovascular hydrostatic pressure and possible variation of the pressure with experimental manipulation (Pandeli et al 1969) and 3) the possibility that some exchange occurs across lymphatics or in transit through nodes so that lymph protein concentration does not reflect perimicrovascular protein concentration. There is considerable evidence that exchange of protein does not occur across peripheral lymphatics (Garlick and Renkin 1970) and lymph nodes (Mayerson et al 1962). Nevertheless, the information that is required would need to be a direct comparative measurement of proteins in interstitial lung fluid and in lung lymph collected at the same time. Technical difficulties have precluded collecting lung interstitial fluid directly to date.

Protein Turnover

Some investigators have tried to assess lung vascular permeability by comparing counts of labeled red cells and of labeled macromolecules accumulating in the lung over time (Aviado 1953, Gorin et al 1975). One study showed that the time course of extravascular ^{113}Indium-labeled transferrin accumulation (estimated by difference between red cell and transferrin counts) was similar with the time course of equilibration in lung lymph.

The concentrations of macromolecules in pulmonary edema fluid have been compared with concentrations in peripheral blood plasma and the time course of the appearance of labeled macromolecules in edema fluid after intravenous injection has been followed (Robin et al 1972). Nevertheless. these studies give only qualitative information about vascular integrity. Because alveolar epithelium is normally completely impermeable to proteins (Taylor and Gaar 1970, Wagensteen et al 1969), the appearance of protein in edema fluid means either that the fluid gets into alveoli by some other route or that the epithelial barrier is damaged. It is not

known whether proteins are partially sieved by the epithelial barrier as fluid goes from interstitium to alveoli, or whether fluid is absorbed or added in transit up the tracheobronchial tree. These problems together with the fact that gross edema fluid is a severe, unsteady perturbation make it difficult to equate edema fluid protein concentrations with protein concentrations in the microvascular filtrate. The ideal would be to sample peripheral blood, interstitial fluid, lung lymph and edema fluid simultaneously.

Radioaerosol Clearance

The rate at which small solutes such as sodium ^{22}Na and sucrose [^{14}C] leave the alveoli after instillation into the bronchus had been used for many years to measure pulmonary permeability (Chinard et al 1962, Egan et al 1976). These invasive techniques measured epithelial permeability in fluid-filled lungs and required multiple samples of alveolar fluid. They are not applicable to awake animals or humans. A preliminary non invasive technique to measure pulmonary epithelial permeability was performed in 1978 (Jones et al 1978). These workers instilled a small volume of a mixture of ^{125}I-antipyrine (molecular weight [MW] 312 daltons) and ^{51}Cr-ethylenediamine tetraacetate (EDTA) (mol wt 377 daltons) into the airways of anesthetized rabbits. Instead of sampling the alveolar space, they measured the rate at which the two labeled molecules entered the blood. They demonstrated convincingly that the rate at which the label appeared in the vascular compartment increased after the integrity of the pulmonary epithelium had been altered by tracheal instillation of hydrochloric acid. Subsequently, a good correlation between the ^{125}I-antipyrine/^{51}Cr-EDTA permeability index and radiographic, functional and postmortem changes in damaged lungs (Jones and coworkers 1979) was revealed. This technique was modified by other investigators (Huchon et al 1981) who took advantage of the simplicity and general availability of technetium-labeled diethylenetriaminepentaacetate (^{99m}Tc-DTPA) to replace ^{51}Cr-EDTA.

Shortly after these preliminary experiments in intact animals, the technique was considerably simplified for use in humans. The ^{99m}Tc-DTPA was delivered as an aerosol, the second tracer was eliminated and the lung clearance of ^{99m}Tc-DTPA was measured by external counting with sodium iodide probes or a gamma camera rather than by blood sampling (Jones et al 1980, Rinderknecht et al 1980). Other workers (Taplin et al 1978, Chopra et al 1979) were some of the first proponents of this technique. Today most investigators use aerosols of ^{99m}Tc-DPTA (MW 492) and sodium pertechnetate (TCO_4^-, MW 163). Some of the aerosol is deposited on the epithelium, the site of which is determined by many variables, the most

important being particle size (Brain et al 1975). Aerosol particles in the size range 1 - 2 μ are produced usually by passing the aerosol through a reservoir bag interposed between the nebulizer and the mouthpiece (Chopra et al 1979, Rinderknecht et al 1980). This allows particles larger than 2μ to settle out in the bag by sedimentation. Various workers have shown that the optimal range of particles for alveolar deposition is 0.3 - 2μm (Chan and Lippman 1980), and as such; particle sizes used in these studies were probably appropriate for alveolar deposition. The deposition sites are therefore distal to the mucociliary escalator and clearance rates could be used as an index of alveolar-capillary membrane permeability. Once deposited, the ^{99m}Tc-DTPA and $^{99m}TcO_4^-$ must traverse the lipid surfactant layer in the alveolus or mucous blanket in the airways before reaching the epithelial cell membrane. It has been assumed that ^{99m}Tc-DTPA remains intact, moves principally through the intercellular junctions (Figure II) and then reaches the lung interstitium and pulmonary vasculature. However, it has been recently suggested that ^{99m}Tc-DTPA may be susceptible to oxidative dissociation in vitro (Nolop 1987) and thus the isotope label may become free before it's translocation across the pulmonary epithelium. For $^{99m}TcO_4^-$ its pseudohalide properties have allowed this solute to move across the airway epithelium implying movement via both the paracellular and transcellular routes (Figure II). In the direction from the air to the blood side this flux has been demonstrated to be a back flux (Man et al 1985). However, the exact mechanisms for translocation of these solutes across the alveolar-capillary membrane are still a matter of great controversy.

The pulmonary epithelium is 5-8 times as restrictive to the movement of solutes as the endothelial membrane; the clearance rate of these radioaerosols therefore reflects the integrity of the epithelium and its covering layer, and not the endothelium. After traversing the epithelium the principal entry of ^{99m}Tc-DTPA to the blood stream is directly into the pulmonary and not bronchial vascular bed (Rizk et al 1984) with little being cleared by the lung lymphatics (Coates and O'Brodovich 1986). Subsequently the disappearance of ^{99m}Tc-DTPA and $^{99m}TcO_4^-$ from the lung is monitored by a sodium iodide detector in a portable probe or a gamma camera. A time activity curve is generated from which the clearance rate constant can be calculated. The rate constant is being measured and not the total amount of transported ^{99m}Tc-DTPA or $^{99m}TcO_4^-$.

One of the major earlier problems with this technique was how to separate activity present in the alveoli and terminal airways during washout from activity in the chest wall. It is well known that the measured clearance rate of any diffusion-limited compound from the lung epithelium will slow if the interstitial and blood levels increase siginificantly. This may be a problem if long monitoring periods are used in subjects with very rapid lung clearance. Some

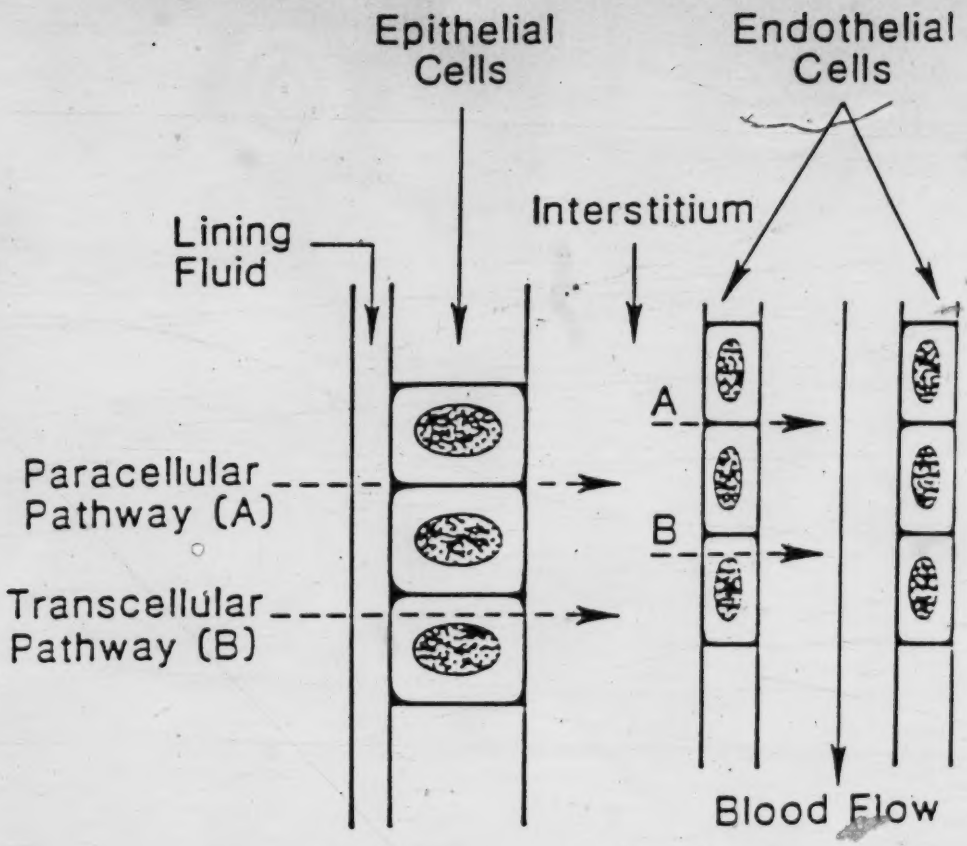


Figure II: Schematic representation of the alveolar capillary barrier and postulated pathways for solute translocation. $^{99m}TcO_4^-$ probably traverses via both paracellular (A) and transcellular (B) pathways and ^{99m}Tc-DTPA via the paracellular pathway alone.

investigators use an intravenous dose of ^{99m}Tc-DTPA and a second probe over the systemic bed (Jones et al 1980, Minty and Royston 1985) to correct for this nonepithelial radioactivity. The validity of this correction procedure is dependent upon its own unproven assumptions; for example, the permeability and perfused surface area of the systemic and pulmonary capillary vasculature and interstitial space are comparable and do not vary with time. Other workers (Jefferies et al *JAP* 1984, Oberderster et al *ARRD* 1986) have not found such corrections necessary.

The major advantage of this radioaerosol scanning technique is that it is easily performed with routine nuclear medicine technology. The clearance rates obtained for normal subjects seem to be fairly reproducible but as expected there are species differences (Jones et al 1983). The technique requires no cooperation from the patient and therefore can be performed on infants and critically ill or otherwise uncooperative patients. It is noninvasive and the only theoretical risk is the exposure to radiation, which is minimal and equivalent to a radioaerosol ventilation lung scan (Coates and O'Brodovich 1984). This method described for measuring alveolar-capillary membrane permeability probably reflects both alveolar-capillary as well as terminal bronchiolar-capillary membrane permeability. To differentiate between the two is very difficult but it is likely that the alveolar component is dominant in view of its greater surface area.

An ideal method for assessing pulmonary permeability has not yet been developed, and the lack of a reliable method that can be used in living animals and humans seriously impedes study of the abnormality in the clinical setting. Criteria for an ideal method would include 1) applicability to living humans and other animals, 2) enough sensitivity to detect increased permeability before pulmonary edema develops, 3) non-invasive character, 4) ability to distinguish between the effects of increased vascular hydrostatic pressure and increased vascular permeability, 5) ability to distinguish between alveolar epithelial and capillary endothelial permeability, 6) quantitative results and 7) speed and reproducibility.

Factors Influencing Pulmonary Clearance Rate

It is clear that the rate of clearance of radionuclide-labeled solutes from the lungs is influenced by changes in pulmonary epithelial permeability to solutes. It is possible however, that other factors might influence pulmonary clearance rates of these compounds. The measured clearance rate could be affected by the site of deposition of the ^{99m}Tc-aerosol, the molecular weight of the solute used, animal species, blood flow, lung volume, lung pressure and surfactant.

Animal Species

A difference in clearance rates for ^{99m}Tc-DTPA has been noted in various species. The slowest clearance rates occur in rats (Minty and Royston 1985), rabbits (Jefferies et al 1984) and sheep (Jefferies et al 1984, O'Brodovich et al 1986). Pigs (Jefferies et al 1984) have clearance rates comparable to humans whereas mongrel dogs (Rizk et al 1984) have the fastest clearance rates.

Site of Aerosol deposition

It has been suggested that different regions of the respiratory tree have different clearance rates for ^{99m}Tc-DTPA. Oberdoster et al (1986) have demonstrated that airways clear ^{99m}Tc-DTPA more slowly than the distal lung units in the dog lung. Others (O'Byrne et al 1984) have observed only a minimally faster clearance rate for ^{99m}Tc-DTPA in the inner hilar regions compared to outer and hence more alveolar lung regions.

The major determinants of regional deposition of aerosols are particle size, rate and depth of ventilation, and regional ventilation (Brain et al 1979). It has been shown (Chamberlain et al 1983) that in adults 80% of a submicronic aerosol of ^{99m}Tc-S-colloid was still in the lungs 24 hours after inhalation. This implies that 80% of the aerosol had deposited beyond the mucociliary transport system, i.e. in small airways and alveoli. Other investigators (Kay et al 1986) have recently demonstrated 80% peripheral deposition of ^{99m}Tc-S-colloid aerosol (AMAD 0.8μ) in small children and new born piglets with both healthy and diseased lungs. Thus, if a submicronic aerosol of ^{99m}Tc-DTPA or $^{99m}TcO_4^-$ is used, the lung clearance technique measures clearance from the small airways and alveoli.

The rate and depth of breathing can also affect the site of aerosol deposition, even with submicronic aerosols (Brain 1979). Therefore it is important that a standard method of inhalation be used to deliver the aerosol. Maximum peripheral deposition occurs during quiet breathing, wheras rapid inhalation causes impaction of aerosol at the bifurcation of major airways and therefore produces a more central deposition. Thus the site of deposition can have a major influence on the clearance rate of ^{99m}Tc-DTPA from the lungs (Oberdoster et al 1986).

Molecular Weight of Solute

The rate of diffusion of small molecules is inversely proportional to the square root of their molecular weights; i.e, the smaller the molecules the faster they diffuse. The exchange of ^{99m}Tc-DTPA from airspace to blood has been suggested as being entirely by diffusion. The molecular weight of ^{99m}Tc-DTPA is 492 whilst that of $^{99m}TcO_4^-$ is 163.

The half time for clearance of $^{99m}TcO_4^-$ in humans is approximately 20 minutes (Rinderknecht et al 1980). Therefore any significant contamination of the inhaled ^{99m}Tc-DTPA aerosol with $^{99m}TcO_4^-$ would therefore produce either a multi-compartment curve or a single-compartment curve with an abnormally short half time. Contamination with colloidal TCO_2 which has a pulmonary biological half-life of days would cause the measured clearance half time to be abnormally long. Chemical breakdown of ^{99m}Tc-DTPA has been reported to occur during nebulization with ultrasonic, but not with jet nebulizers (Waldman et al 1985).

Lung Volume and Pressure

It has been demonstrated that the clearance rate of $^{99m}TcO_4^-$ from the upper lobes of erect volunteers was faster than from the lower lobes (Rinderknecht et al 1980). Those workers postulated that the more rapid clearance from the upper lobes was due to higher alveolar volume in those regions and not a decreased apical blood flow. Their suggestion has been supported by the elegant work of Rizk et al (1984) who demonstrated the trivial effect of changes in pulmonary-artery blood flow on the pulmonary clearance of ^{99m}Tc-DTPA in dogs.

Several groups have reported that positive end expiratory pressure (PEEP) or continuous positive airway pressure (CPAP) increases the ^{99m}Tc-DTPA clearance rate from the lungs (Mason et al 1984, Nolop et al 1985). There appears to be a threshold effect with a minimal acceleration of clearances seen with PEEP less than 10 cm H_2O, but marked acceleration of clearance at a PEEP of 12-18 cm H_2O (Marks et al 1985, O'Brodovich et al 1986). This effect of PEEP and CPAP is secondary to the increase in alveolar volume produced by these maneuvers. As evidence for this, two independent groups have shown that human volunteers have a similar increase in ^{99m}Tc-DTPA lung clearance rate at high lung volumes, regardless of whether the increase in volume is produced by CPAP or by voluntary muscle effort (Nolop et al 1985, Marks et al 1985). More recently other investigators (Man et al 1987) have demonstrated that high frequency oscillatory ventilation (HFOV) increases clearance rate of ^{99m}Tc-DTPA.

Altering the pattern of breathing during inhalation of the aerosol will affect its site of distribution (Brain et al 1979). Fortunately, alterations in breathing pattern that affect aerosol deposition do not affect clearance rates. For example, increased breathing frequency (Rizk et al 1984) or increased inspiratory resistance with markedly negative pleural pressures (O'Brodovich et al 1986) has no effect on the clearance rate of ^{99m}Tc-DTPA from the lungs in sheep.

The mechanisms for the increase in clearance of ^{99m}Tc-DTPA with PEEP, CPAP, or HFOV is not known but it has been suggested that it could be due to a) an increase in alveolar surface area available for diffusion by unfolding the alveolar pleats (Rizk et al 1984), b) increasing the permeability of the epithelium by "stretching" the interepithelial junction (Mason 1984) or c) affecting the functional integrity of the alveolar surfactant layer and permitting a more rapid access of the lipophobic saline droplet to the entire underlying hypophase and epithelium. hence increasing the surface area available for diffusion (Oberdoster et al 1985).

Surface lining of the Respiratory tract

The ciliated airways are covered by a mucous blanket. It is not known whether this creates any barrier to the in vivo diffusion of ^{99m}Tc-DTPA or $^{99m}TcO_4^-$ through the epithelial membrane and into the blood stream. Equally important is the potential influence of the surfactant layer lining the distal lung unit. This phospholipid protein complex has its hydrophobic region directed into the air of the alveolar space. The ^{99m}Tc-DTPA contained within the submicronic hydrophilic and lipophobic saline aerosol could be slowed in its movement through the alveolar capillary membrane by the surfactant in 2 different ways. Firstly, it could diffuse more slowly and secondly, the droplet could be prevented from rapidly reaching and spreading throughout the alveolar liquid hypophase.

Experimental data support the speculation that the alveolar surface tension characteristics can influence ^{99m}Tc-DTPA clearance. These include the observation that hyaline membrane disease, a disorder initiated by a relative surfactant deficiency is characterized by an extremely rapid clearance rate of ^{99m}Tc-DTPA (Jefferies et al 1984). Similarly, saline lavage surfactant depletion accelerates ^{99m}Tc-DTPA clearance from adult rabbit lungs (Wollner et al 1986).

5. CONDITIONS ASSOCIATED WITH ALTERED RADIOAEROSOL CLEARANCE

Acute Lung injury

The clearance rate of ^{99m}Tc-DTPA appears to be similar after a normal birth and during adult life (Jefferies et al 1984). When healthy, both right and left lungs clear ^{99m}Tc-DTPA at similar rates (Meignam et al 1986).

In general, acute lung injury leads to the development of increased epithelial and endothelial permeability. The two most common acute lung injury syndromes are infantile respiratory distress syndrome or hylaine membrane disease and the adult respiratory distress syndrome (ARDS). Both of these disorders have been demonstrated to result in extremely rapid clearances of ^{99m}Tc-DTPA during the acute stages of the illness, with clearances returning towards or to normal values as the subject's condition improves (Jefferies et al 1984, Mason et al 1985). Similarly, fast clearances have been reported in a small group of patients who developed respiratory failure associated with pulmonary infection following bone marrow transplantation (Braude et al 1985). Both of the lung injury syndromes were characterized by alveolar pulmonary edema and it is unlikely that the rapid clearance was occurring from flooded alveoli, Rather the rapid clearance must have occurred either from abnormal but less damaged non-fluid filled alveoli or from distended and damaged alveolar ducts and terminal bronchioles that characterized both these disorders.

A variety of experimental models of acute lung injury have also been characterized by rapid clearances of ^{99m}Tc-DTPA . These include airway instillation of hydrochloric acid (Jefferies et al 1984, Jones et al 1978) and intravenous injection of oleic acid (Huchon et al 1981, Jefferies et al 1984). The rapid clearance of ^{99m}Tc-DTPA in these acute lung injury syndromes has been interpreted as indicating an increase in pulmonary epithelial permeability to solutes in the alveolus itself. Although this is the most likely explanation some workers (O'Brodovich and Coates 1987) emphasized the fact that rapid clearance of ^{99m}Tc-DTPA may reflect other aspects of acute lung injury; for example, deficiency or dysfunction of the surfactant layer, overdistended and damaged alveolar ducts and bronchioles, or reflect the presence of oxidizing agents on basement membranes.

Inhaled gases

It has been demonstrated in multiple studies that young asymptomatic cigarette smokers have rapid lung clearances of ^{99m}Tc-DTPA (Jones et al 1980, Huchon 1984, Mason et al

1983). Accelerated clearances of ^{99m}Tc-DTPA have resulted from exposing nonsmoking humans (Minty et al 1984) and rabbits (Witten et al 1985) to cigarette smoke. Clearance in both humans (Huchon et al 1984) and rats (Minty et al 1985) return to normal upon cessation of exposure to cigarette smoke. The particulate matter and not the gaseous phase or nicotine in smoke results in the increased clearance rate observed in rats (Minty et al 1986), but it is unknown what mechanisms are involved. Potential explanations include an interference with the surfactant within the alveolus, primary oxidant effects, or stimulation of intrapulmonary inflammation by local vasoactive mediators or effector cells. Interestingly, it has been demonstrated that if patients with chronic obstructive lung disease have stopped smoking a normal clearance of ^{99m}Tc-DTPA is observed (Huchon et al 1984).

Ozone, a major component of photochemical smog has been shown to alter the permeability properties of the respiratory epithelium of guinea pigs (Hu et al 1982, Davies et al 1980). For example, after 3 hours resting exposure to as little as 0.26 ppm ozone, guinea pigs have increased protein concentration in their bronchoalveolar lavage fluid, presumably evidence of an increase in permeability of their respiratory epithelium (Hu et al 1982). A more recent study using 8 healthy, nonsmoking adults showed that ozone increased the clearance rate of ^{99m}Tc-DTPA (Kehrl et al 1987).

NO_2, an oxidant gas has also been shown to alter the permeability properties of the respiratory epithelium. However, in contrast to other pulmonary clearance studies of ^{99m}Tc-DTPA (ozone (Kehrl et al 1987) and cigarette smoke (Minty et al 1984)), exposure to 5 ppm NO_2 at normal tidal volumes caused no change in clearance of ^{99m}Tc-DTPA. However, when tidal volumes were increased the clearance of ^{99m}Tc-DTPA was decreased (Oberdorster et al 1986).

These studies therefore suggest that the pulmonary clearance of radioaerosols may be a useful approach for assessing the effects of reactive airborne pollutants upon respiratory epithelium.

6. BRONCHOALVEOLAR LAVAGE AND ITS APPLICATION TO INHALATIONAL LUNG INJURY

Over the past decade as fiberoptic bronchoscopy has become an increasingly used tool in the evaluation of airway and parenchymal lung disease, bronchoalveolar lavage (BAL) has emerged as a valuable technique for sampling cellular and soluble components of the lower respiratory tract for clinical and research purposes (Hunninghake et al 1979, Young and Reynolds 1984). While fiberoptic bronchoscopy has been in widespread use in Japan, the United States and Europe since the 1960's, direct cannulation of the lower respiratory tract dates to the mid-19th century, when Green's report of bronchial catheterization was met with scorn and derision (Gee and Fick 1980). Chevalier Jackson of Philadelphia pioneered the practical use of rigid bronchoscopy in the 1920's for airway examination and removal of foreign bodies. In 1922, bronchial lavage was used to treat a patient with phosgene gas poisoning (Gee and Fick 1980). Approximately 50 years ago the practice of washing out a portion of the lung to remove accumulated secretions or to treat intractable asthma was first practiced (Crystal et al 1986).

The list of noncellular components identified in BAL fluid is long and ever increasing. Most of these substances are proteins or lipids. They represent both materials that transude from the serum and substances that may be synthesized locally in the lung. Many of the proteins identified in BAL are important in protecting the lung from assault by digesting enzymes (e.g. alpha-1-antiprotease). Proteins in respiratory secretions come from two major sources: transudation across the barrier between the capillaries and the alveolar epithelial surface (blood-air barrier), and local synthesis by cells lining the bronchial and alveolar epithelial surfaces. The amount of protein that transudes from blood into the epithelial lining fluid of the lung depends on the size of the particular protein in question, its electric charge and molecular configuration, and the integrity of the alveolar-capillary barrier. The integrity of this barrier may be affected by such diverse factors as hydrostatic pressure in the pulmonary capillaries, oxidizing gases and damage from inflammatory processes occurring on either the endothelial surface of the capillary or the epithelial surface of the lung. Substances of low molecular weight (e.g. electrolytes, sugars, urea) and similar proteins such as albumin (molecular weight approximately 69,000) pass relatively unhindered across the normal endothelial barrier. There appears to be little impairment of diffusion for proteins up to molecular weights of approximately 200,000 while above this there is a significant restriction of transudation (Young and Reynolds 1986).

A number of investigators have used various techniques (including measurement of radiolabeled albumin, influx of erythrocytes and clearance of dextran molecules of known size

into the airways) to measure alveolar capillary permeability in experimental models and human ARDS (Sibbaldi et al 1986). The principal protein identified in BAL fluid is albumin, which accounts for approximately 30% of the total protein identified. Since albumin is synthesized solely by the liver and enters the epithelial lining fluid of the lung only by transudation, it provides a useful marker of serum protein entry into the lung and is frequently used as the denominator against which concentrations of other proteins are normalized.

An important problem in the interpretation of lavage findings, lies in the fact that the amount of alveolar epithelial lining fluid sampled in a given BAL procedure is unknown and there is no reliable denominator against which amounts of proteins and numbers of cells may be standardized. For this reason protein concentrations are frequently expressed in relation to the amount or concentration of albumin retrieved and cell counts are expressed in absolute numbers or in number of cells per volume of lavage fluid recovered.

In order to circumvent this difficulty and to estimate the relative dilution of the epithelial lining fluid (ELF) by BAL fluid, urea has been used as a marker of dilution (Rennard et al 1986). By measuring urea concentrations in plasma and BAL fluid and assuming that the concentration of urea in the ELF equals that of plasma and that the diffusion of urea into BAL fluid is negligible during the short duration of the procedure these investigators were able to estimate the volume of ELF recovered. However the utility of the urea method for calculating ELF volume has been questioned by other workers (Marcy et al 1987). They found that while there was a successive decrease in total protein and IgG concentrations in serial BAL aliquots, the concentration of urea increased, suggesting that the diffusion of urea into BAL fluid was not negligible and may introduce significant artifacts into the estimation of ELF volume.

Recovery of instilled BAL fluid and protein and cellular components can be influenced by the specific details of the BAL protocol used (Crystal et al 1986). Some of the factors influencing BAL recovery of soluble and cellular components are listed in Table 2.

Table 2: FACTORS INFLUENCING BAL RECOVERY OF SOLUBLE AND CELLULAR COMPONENTS

Temperature, pH, divalent cation content of instilled fluid
Aliquot size and number: total instilled volume
Presence of obstructive lung disease
Processing of lavage material
  Number of centrifugations in washing
  Cytocentrifuge versus filter preparations for differential counting
  Method of concentration of fluid phase of BAL

Table 2 continued:

Trauma to airways

Adequacy of wedge of bronchoscope in airway

General aspects of the application of BAL to the quantitation of airway and parenchymal damage have been carefully reviewed (Brain and Deck 1985, Henderson et al 1984). These workers have demonstrated that the utility of BAL as a means of assessing respiratory tract injury from toxic inhalation relies on the accumulation of inflammatory cells and the release of cellular and serum constituents into the epithelial fluid following an inhalational injury.

Henderson et al (1984) have systematically reviewed the various constituents of BAL fluid that may be used to assess airway damage. They have shown that total protein may rise in response to damage to the alveolar-capillary membrane with subsequent transudation of serum proteins into the epithelial lining fluid of the lung. Cytoplasmic enzymes (including lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase, glutathione peroxidase, and glutathione reductase) are released when there is significant damage to cell membranes. Changes in cellular populations in BAL may also be used as indicators of lung injury, with increases in neutrophils and/or macrophages being seen in both acute and chronic inflammatory reactions.

Exposure to oxygen in pharmacologic concentrations is well known to be toxic to the lung (Deneke and Fanburg 1980; Frank and Massaro 1980). A number of investigators have used BAL methodology to study the pathogenesis of pulmonary oxygen toxicity. Fox and colleagues (1981) investigated the mechanism leading to accumulation of neutrophils in the lung of rats exposed to 100% oxygen. They noted that chemoattractant activity in the lavages of rats was increased and correlated closely with neutrophil counts in BAL from exposed animals. However, pretreatment of animals with cobra venom factor to deplete them of complement did not alter the results which suggested that products of complement activation were likely not the only chemoattractant substances present in their model.

BAL has also been used to examine changes in the permeability of the alveolar capillary membrane and the factors secreted by bronchoalveolar cells after exposure of normal human subjects to 95% oxygen for 17 hours (Davis et al 1983). That report noted increased amounts of albumin and transferrin in BAL fluid, suggesting an increase in permeability of the blood-air barrier in response to hyperoxic exposure.

In contrast to the well recognized adverse clinical effects of very high inspired oxygen tension, conventional clinical wisdom has deemed lower concentrations (21 - 50% FiO_2) to be safe. Griffith and colleagues (1986) performed baseline and followup on BAL albumin and

^{99m}Tc-DTPA clearance studies on normal individuals before and after exposure to normal "therapeutic" oxygen concentrations. They observed that permeability of the blood air barrier as judged by BAL albumin concentration was increased after breathing concentrations of oxygen of 30% or higher for a mean duration of 45 hours. Lung clearance of the isotope was significantly increased in the group who breathed 50% oxygen and in these individuals there was a trend toward an increased percentage of BAL neutrophils. In none of the individuals was there bronchoscopic evidence of lung inflammation or biochemical evidence of lung tissue injury as judged by BAL concentrations of LDH, alkaline phosphatase, potassium or several eicosanoids.

Very few studies have evaluated the bronchoalveolar lavage technique in NO_2 inhalational injury. However, one group of workers (Sherwin and Richters 1971) has demonstrated increased lung capillary permeability in mice by injecting them intravenously with tritiated albumin before and at intervals after NO_2 exposure and then analyzing pulmonary lavage fluid. A few years later Sherwin and Layfield (1976) showed that the protein content of homogenized lung tissue of mice exposed to 0.5 ppm NO_2 for 10 - 14 days was increased.

Bronchoalveolar lavage has therefore provided a sensitive, reproducible and relatively non-invasive means of sampling the cellular and humoral milieu of the lower respiratory tract and it will therefore find an increasingly important place in the armamentarium of the inhalational toxicologist in studying lung injury induced by inhaled substances in both human subjects and experimental animals.

7. PULMONARY MICROCIRCULATORY METABOLIC FUNCTION

There has been extensive study of several nonrespiratory functions of the lung. Many studies have focused on the ability of the lung to remove and/or metabolize various important vasoactive hormones (eg. prostaglandin, biogenic amines and peptides) from blood entering the pulmonary circulation. Most of these metabolic processes reflect functions of microvascular endothelial cells of the lung (Junod 1975, Gillis and Roth 1976). Thus, if endothelial damage is a frequent response of the lung to injury it seems logical to anticipate simultaneous changes in one or more of the metabolic functions of these cells. It has been proposed that should changes in nonventilatory endothelial metabolic functions precede altered permeability of the alveolar-capillary membrane, it may provide the basis for development of new techniques aimed toward detection of lung injury at its earliest stages.

It is well known that lungs of experimental animals and man are capable of affecting the disposition of several blood-borne vasoactive substances. These metabolic functions of lung include the capacity to 1) transport and metabolize prostaglandins of the E and F series and several biogenic amines including 5-hydroxytryptamine and norepinephrine, and 2) hydrolyze several peptides including Angiotensin I and bradykinin via activity of angiotensin converting enzyme. These saturable drug and temperature sensitive processes are associated with the pulmonary endothelium. Study of these functions can yield substantial, important information about the biochemical integrity of the pulmonary microcirculation.

A variety of techniques and preparations have been used to investigate the fate of biogenic amines and prostaglandins in the lung and pulmonary circulation after lung injury. These include distribution studies in the whole animal, measurement of inactivation of substrates by homogenized lung preparations and measurement of the disappearance of substrate from the pulmonary circulation in-vivo or in isolated perfused lungs. Distribution studies where the substrate concentration in lung tissue is determined at varying times after administration, do not reflect the ability of the lung to remove substrate in one circulation time, since the lapse of time allows redistribution to take place. Similarly, studies in homogenized or minced lung tissue do not necessarily reflect the metabolizing activity of the pulmonary circulation, since homogenization of tissue can expose enzyme systems not normally accessible to the substrate on passage through the pulmonary circulation. Removal of a substrate in one circulation through the lungs in vivo is measured by injecting it intravenously or into the right heart and determining the amount surviving passage through the pulmonary circulation by either serial sampling of arterial blood and estimation of the substrate chemically or biologically or by continuous bioassay using the blood-bathed organ technique (Vane 1964, 1969).

In these in-vivo experiments as in isolated lung preparations, the quantitative

determination of removal of an amine or prostaglandin depends on a) whether the substrate is in the lung effluent at fixed time intervals or b) assaying peak biologic activity. In addition results depend on whether the amine is administered as a single bolus injection where a high concentration is attained for a short time, possible temporarily overwhelming an uptake system, or as an infusion and removal assessed under steady state conditions. For example, the pulmonary circulation of dogs in vivo removed only 33% of serotonin (5 HT) injected into the pulmonary artery (Davis and Wang 1965) whereas up to 98% of intravenously infused 5 HT was inactivated (Thomas and Vane 1967).

Isolated lungs perfused through the pulmonary artery offer perhaps the best method for investigating the properties of the pulmonary uptake and metabolism systems for biogenic amines and prostaglandins since external factors can be varied and controlled to a greater extent than in whole animal experiments and a lower concentration of substrate can be used. The concentration used is an important factor when the substrate under study can cause marked changes in pulmonary blood flow and when studying an uptake system that can be saturated. This has been clearly illustrated (Boileau and Assosciate (1972) in experiments where lung removal of nonadrenaline in the anesthetized dog decreased from 54% to 5% as the infusion concentration increased from 0.2 to 3 mg/kg/minute. In studies of pulmonary uptake in isolated perfused lungs, biogenic amines are administered via the pulmonary artery and measurements are made of either the amount of biogenic amine retained by the lung or the amount of amine surviving passage through the lung.

Studies in anesthetized animals (Davis and Wang 1965, Catravas and Gillis 1980) and in isolated blood-perfused lung preparations (Rickaby et al 1981) have shown that there is an efficient removal process for 5 HT in the pulmonary circulation. Other investigators, (Strum and Junod 1972), using radioautography observed that the uptake of ^{3}H labeled 5 HT by the isolated lung occurred almost exclusively in pulmonary endothelial cells. Moreover the uptake process has been demonstrated to be inhibited by tricyclic antidepressents (Catravas et al 1980). Junod (1972) found that ^{14}C-5HT uptake by the lung at higher concentrations approached a constant rate, suggesting a saturable process. These characteristics of specific inhibition and saturability of 5 HT uptake satisfy criteria for a mediated-transport process. Pulmonary uptake of 5 HT also appears to be an energy requiring process since the removal is very dependent on temperature. In rat lungs, lowering the temperature of the perfusing fluid from 37° to 4° produced an 86% inhibition of ^{14}C-5 HT removal (Junod 1972). Furthermore, it has been shown that a sodium carrier system is probably involved in the transport of 5-HT across the cellular membrane in the pulmonary circulation, since lung removal of 5-HT was inhibited by a lowered sodium concentration in the perfusing fluid (Junod 1972). Thus it is

postulated that the uptake of 5 HT is a sodium-dependent, carrier-mediated transport.

Since Ferreira and Vane (1967) first reported that over 90% of the biological activity of intravenously injected prostaglandins E_1, E_2, or E_3 was lost during passage through the pulmonary circulation, a number of investigators have shown that radiolabeled prostaglandins of the E series (Dawson 1975, Hook 1975) are rapidly taken up and metabolized in isolated perfused lungs. It has been demonstrated (Hammond et al 1977) that ^{3}H-prostaglandin E_1 is cleared by the lungs of patients undergoing cardiopulmonary bypass. Moreover, (Klein et al 1978) exposure of rats to 100% O_2 for 36 and 48 hours resulted in a decreased ability of the isolated rat lung to metabolize prostaglandin E_2. Similarly, in adult patients with ARDS the pulmonary removal of ^{3}H-PGE_1 and 5-HT was noted to be depressed (Gillis et al 1986). Thus it has been postulated that changes in endothelial cell function may precede measureable gross pathophysiological changes which were manifested in the frequently irreversible phases of ARDS. Furthermore, damage to pulmonary endothelium of experimental animals after exposure to high concentrations of oxygen, and bleomycin are all associated with depressed removal of prostaglandins or amines (Block 1977, Catravas et al 1983).

Alterations in pulmonary blood flow, perfusion heterogeneities, mean transit time and endothelial cell surface area as well as the integrity of the transport and/or enzymatic properties of the pulmonary microcirculations can influence the disposition of vasoactive substances. Factors that may influence removal of substrates by lung include 1) Concentration of substrate in blood, 2) Lipid solubility of the substrate, 3) Diffusional characteristics of the substrate, and 4) Degree of protein binding and/or ionization of substrate. When low amounts of substrate are used , conditions of excess carrier or enzyme exist which frequently lead to relatively small changes in pulmonary metabolism with gross pathologic or hemodynamic perturbations. Furthermore, these two types of disruptions of the pulmonary microcirculation may not be readily distinguishable as they affect the metabolic disposition of low amount of substrate.

The term uptake refers strictly to the transfer of substrate across a cell membrane, and the true rate of uptake cannot be measured unless the substrate is retained in the tissue without significant leakage or enzyme inactivation. Since pulmonary metabolism of certain biogenic amines and prostaglandins involves both an uptake an enzyme activation process, the term pulmonary removal will be used in this thesis to describe reduction in biologic activity or concentration of amine or prostaglandin during passage through the lungs.

8. EXPERIMENTAL

Introduction

Nitrogen dioxide (NO_2), an oxidant gas, is a major pollutant in urban environments. In the airways, acute exposure to NO_2 results in altered permeability as evidenced by transudation of serum proteins from the vascular space (Denicola et al 1981), and penetration into epithelium of high molecular weight tracers (Ranga et al 1980). As well, acute toxic inhalation of NO_2 has been shown to cause morphologic and functional changes of the lungs that include interstitial edema, recruitment of inflammatory cells and impaired surfactant activity (Dowell et al 1971, Guidotti 1978). The primary sites of lung injury are the terminal bronchioles (Guidotti 1978, Parkinson and Stephens 1973) and the alveoli and alveolar ducts distal to these airways. The precise mechanism of NO_2-induced injury is unknown. However, one of the major postulates involves free radical-mediated peroxidative cleavage of membrane lipids (Mustafa and Teirney 1978). In order for pulmonary edema to occur it has been suggested that the barrier function of the capillary endothelium must have been altered. But, the permeability of the pulmonary epithelium has not yet been addressed.

Recently, interest has been focused on the role of altered permeability of the alveolar-capillary membrane in inhalational lung injury. Much of the literature has centered on the translocation of solutes and most studies have evaluated cigarette smoke (Jones 1980) and oxygen (Erdmann et al 1983, Matalon and Egan 1981). One approach to the evaluation of increased permeability has been the study of clearance of radiolabeled aerosols; in particular ^{99m}Tc-DTPA and $^{99m}TcO_4^-$ (Rinderknecht et al 1980, O'Brodovich and Coates 1987). Another approach has been the application of bronchoalveolar lavage to examine changes in the permeability of the alveolar-capillary membrane by measuring cellular and noncellular products of inhalational lung injury (Davis et al 1983, Henderson 1984). On the other hand lung vascular permeability in vivo has been evaluated by the indicator dilution technique (Brigham and Snell 1973).

It has been noted that ^{99m}Tc-DTPA clearance was not altered at normal tidal volumes in dogs exposed to 5 ppm NO_2 for 1 hour and in fact was decreased at high tidal volumes after NO_2 (Oberdorster et al 1986). Nevertheless, it has been demonstrated that tight junctions are altered after NO_2 exposure; implying that alveolar epithelial permeability is affected (Gordon et al 1986). With respect to the capillary endothelium it has been recently shown that 5 ppm NO_2 reduced 5HT uptake in cultured endothelial cells after 24 hours exposure but not after 3 or 12

hours exposure (Patel and Block 1986). Thus it is unclear whether alveolar permeability changes precede, occur simultaneously with metabolic function alterations, or occur as a result of cell injury. Moreover, it has been difficult to determine how the permeability changes interrelate with other lung function parameters.

In this study, two aerosolized radiolabeled markers, sodium pertechnetate ($^{99m}TcO_4^-$) and technetium-labeled diethylenetriaminepentaacetate (^{99m}Tc-DTPA), with different physicochemical properties were used. These solutes have been used to assess lung epithelial permeability in both humans and animals (Mason et al 1983, Rinderknecht et al 1980, Ahmed et al 1986). One major limitation of their use, however, is the lack of definitive information concerning the pathways by which these solutes travel across the alveolar surface and into the blood.

The concentration of albumin in the bronchoalveolar lavage (BAL) fluid has also been used as a function of epithelial permeability (Goren and Stewart 1979). The specificity of these methods (especially BAL albumin concentration) in detecting permeability changes is not yet established, but it is likely that permeability of the alveolar-airway epithelium has a major influence on both measurements. Using a BAL technique albumin concentration and cellular content of BAL fluid were evaluated in the dog after compressed air and NO_2 exposure.

It has been well documented that nonrespiratory metabolic functions of the pulmonary microcirculation, particularly removal from the circulation of some substances such as prostaglandin E and serotonin, are depressed in experimental injury caused by hyperoxia and intra-tracheal bleomycin instillation (Block and Fisher 1977, Catravas et al 1983). It has therefore been proposed that depressed pulmonary extraction of these compounds might provide a means for the detection of early injury to the pulmonary endothelium. Using a multiple indicator dilution technique (Gillis et al 1979) pulmonary removal of ^{14}C-5hydroxytryptamine (^{14}C-5HT) and ^{3}H-prostaglandin E_1 (^{3}H-PGE_1) was evaluated in the dog before and after an acute NO_2 exposure; both compounds were used in trace amounts to eliminate their profound effects on the hemodynamic states of the animal.

NO_2 has also been shown to alter pulmonary mechanics (Bylin et al 1985, Speizer et al 1980) and it has been demonstrated that changes in lung volume also affect alveolar clearance of the radioaerosol (Marks et al 1985). In order to evaluate changes of clearance of ^{99m}Tc-DTPA and $^{99m}TcO_4^-$ after NO_2 inhalation, functional residual capacity (FRC) measured using a helium dilution technique and static lung compliance (CL), were obtained in some animals. As well, to provide a morphological basis for the correlation with the physiological changes, animals were sacrificed at different time intervals after NO_2 inhalation for histological

assessment of the lung.

This study was designed to assess the magnitude and reversibility of the NO_2 effect. It was felt that a documentation of the sequential changes of these functions would provide much needed information in understanding the pathogenesis of NO_2 induced lung injury.

Methods

Nineteen adult mongrel dogs of either sex weighing 13-23 kg were used in the study. Two concentrations of NO_2 were used (200 ppm and 400 ppm); these gases were obtained premixed and certified from Matheson Gas, Edmonton, Alberta. The duration of exposure to either concentration was 60 minutes and the gas was delivered to the animal via an endotracheal tube. The animal was anesthetized with pentobarbitol sodium, at a dosage of 30mg/kg and breathed spontaneously throughout the study. Metabolic function of the pulmonary microcirculation, pulmonary epithelial permeability, functional residual capacity and static compliance were measured in vivo (Table 3). Two control measurements on compressed air (ctrl) were carried out one hour apart to show the reproducibility of the studies. To study the sequential recovery or changes due to a specific concentration of NO_2, measurements were repeated after NO_2 exposure at 1 hour, 2 days and 7 days for both NO_2 concentrations (200 ppm and 400 ppm), and for 400 ppm NO_2 only, also at 14 days. Nine dogs received 200 ppm and 4 received 400 ppm NO_2. Eleven dogs were studied with radioaerosol scans.

Because of the length of the procedures, not all animals underwent all the tests. Several animals were sacrificed at different times after NO_2 inhalation. The sequence of the study was measurement of pulmonary mechanics, multiple indicator dilution for metabolic function of the pulmonary microcirculation, and followed by radioaerosol study.

Six different dogs, two groups of three, each were used to measure BAL albumin concentration and cell content. The first group received compressed air and the second group NO_2 at 200 ppm for one hour on day one. Fourteen days later the inhalation history was reversed with the first group receiving NO_2 and the second compressed air. Bronchoalveolar lavage was performed approximately 90 minutes after the cessation of exposure to compressed air or NO_2 on day one and day fourteen in order to coincide with the time that the ^{99m}Tc-DTPA scan had been measured in the other group of dogs (Figure III).

Table 3

Animals	200 ppm	400 ppm	Aerosol	Removal	CL	FRC	Histology
G330	X			X	X		X
G373	X		X	X	X		
G302	X		X	X	X		X
G422		X	X	X	X		X
H6		X	X			X	X
H54		X	X			X	X
H75	X		X	X		X	X
H17	X		X			X	
G479	X		X	X			X
F197	X			X			
F358		X	X	X			
F419	X		X				
H98	X		X				
Totals:	9	4	11	8	4	4	7

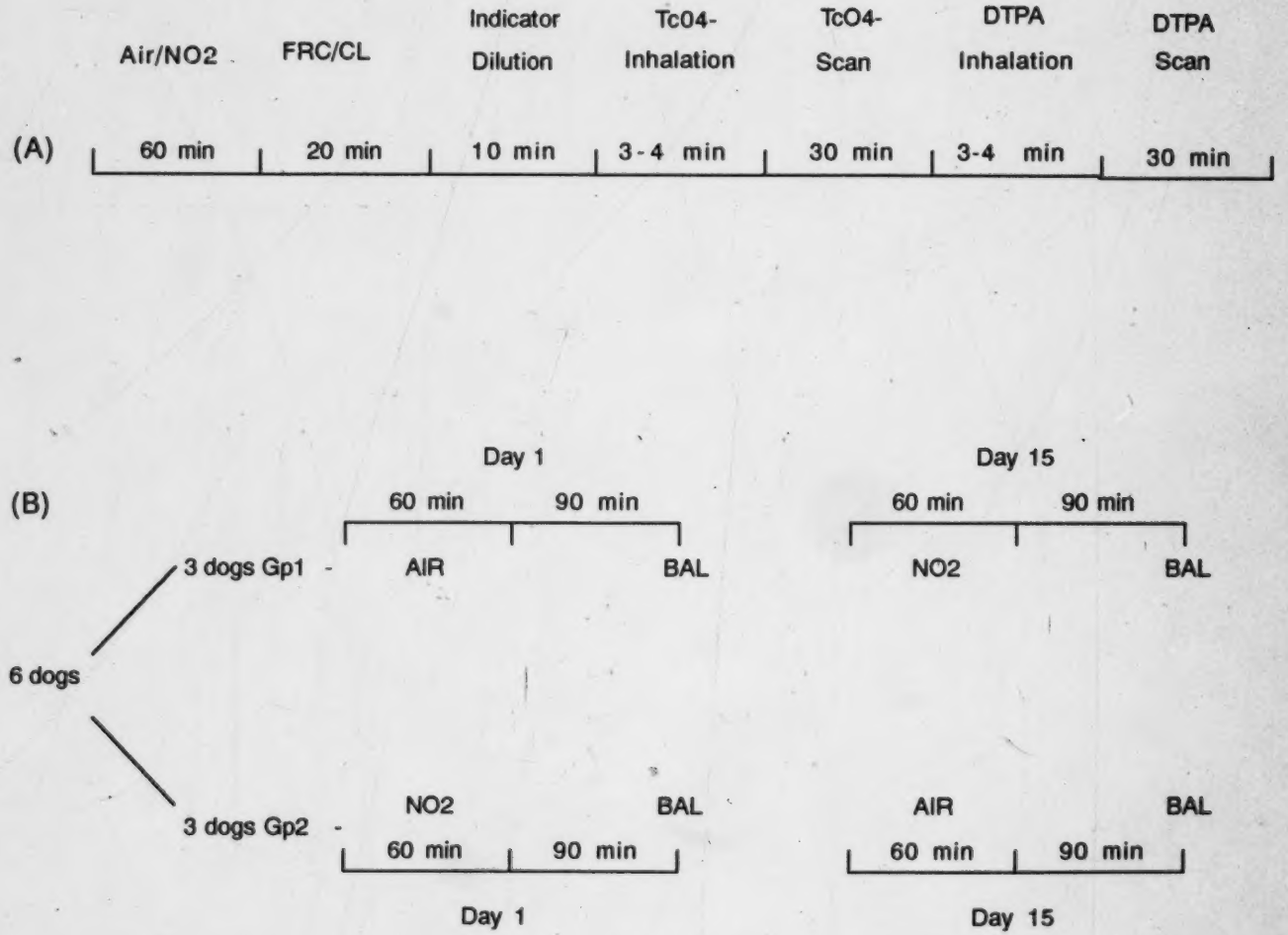


Figure III - Experimental protocol. (A) For 13 dogs undergoing scanning, lung mechanics and indicator dilution procedures. (B) For dogs undergoing bronchoalveolar lavage (see text for explanation).

Radioaerosol Scanning

Pulmonary epithelial permeability was assessed using a radioaerosol technique, in which clearance rates of $^{99m}TcO_4^-$ and ^{99m}Tc-DTPA were measured. The $^{99m}TcO_4^-$ scan always preceded that of the ^{99m}Tc-DTPA. Both scans were assessed for 30 minutes. A similar experimental set-up for aerosol inhalation and gamma scanning has been previously described (Ahmed et al 1986). In brief, the intubated and anesthetized dog was placed supine with the ventral chest wall in close apposition to the face of a gamma camera (Pho Gamma IV, Siemens, Pointe Claire, Quebec, Canada) Figure IV. ^{99m}Tc-DTPA was freshly prepared prior to each scan by adding $^{99m}TcO_4^-$ (815 MBq in 2 ml) to a DTPA kit (Frosst, Montreal, Canada). Thin layer chromatography of the freshly prepared ^{99m}Tc-DTPA solution, the aerosol particles and the solution remaining in the nebulizer at the end of aerosolization, showed ^{99m}Tc-DTPA to be greater than 99.5% bound in four randomly selected experiments. Before radioaerosol inhalation the dogs were given 3 manual hyperinflations (30 ml/kg) over a period of 1 minute. Aerosols were generated from 2 ml of the solution (600 MBq of $^{99m}TcO_4^-$ or 815 MBq of ^{99m}Tc-DTPA) placed in a Hudson disposable nebulizer operating at 35 psi air pressure and 10 L/minute air flow. An 8 liter bag interposed between the nebulizer and the dog removed large particles so that particles delivered to the animal had an activity mean aerodynamic diameter (AMAD) of 1.54 μm +/- 1.88 (geometric standard deviation) when samples were sized with a cascade impactor (Andersen Samplers, Atlanta, GA). No difference in particle size was noted between the two radioisotope solutions.

The dogs inhaled the aerosol spontaneously via a number nine cuffed endotracheal tube for 2-3 minutes. A total lung count rate of 30,000 cpm was obtained which was at least 15 times background. Changes in radioactivity during inhalation and thereafter were recorded by a gamma camera interfaced to a PDP 11/34 computer system (Digital Equipment Hudson, MA)(Fig.IV). The right and left lungs were analyzed as two different regions of interest (ROI) and the central airways were completely excluded. Counts were flood, decay and background corrected and clearance curves of radioactivity against time were plotted. For $^{99m}TcO_4^-$ and ^{99m}Tc-DTPA, $t_{½}$ of the curve over 10, 20 and 30 minutes was calculated using an exponential decay in a manner similar to that used by other investigators (Rinderknecht et al 1980). Blood background determination using a modified method (Jones et al 1983, Yeates et al 1973) was performed on 3 occasions following NO_2 inhalation. It was found that these values did not alter the overall calculation of $t_{½}$ for either ^{99m}Tc-DTPA or $^{99m}TcO_4^-$ clearance.

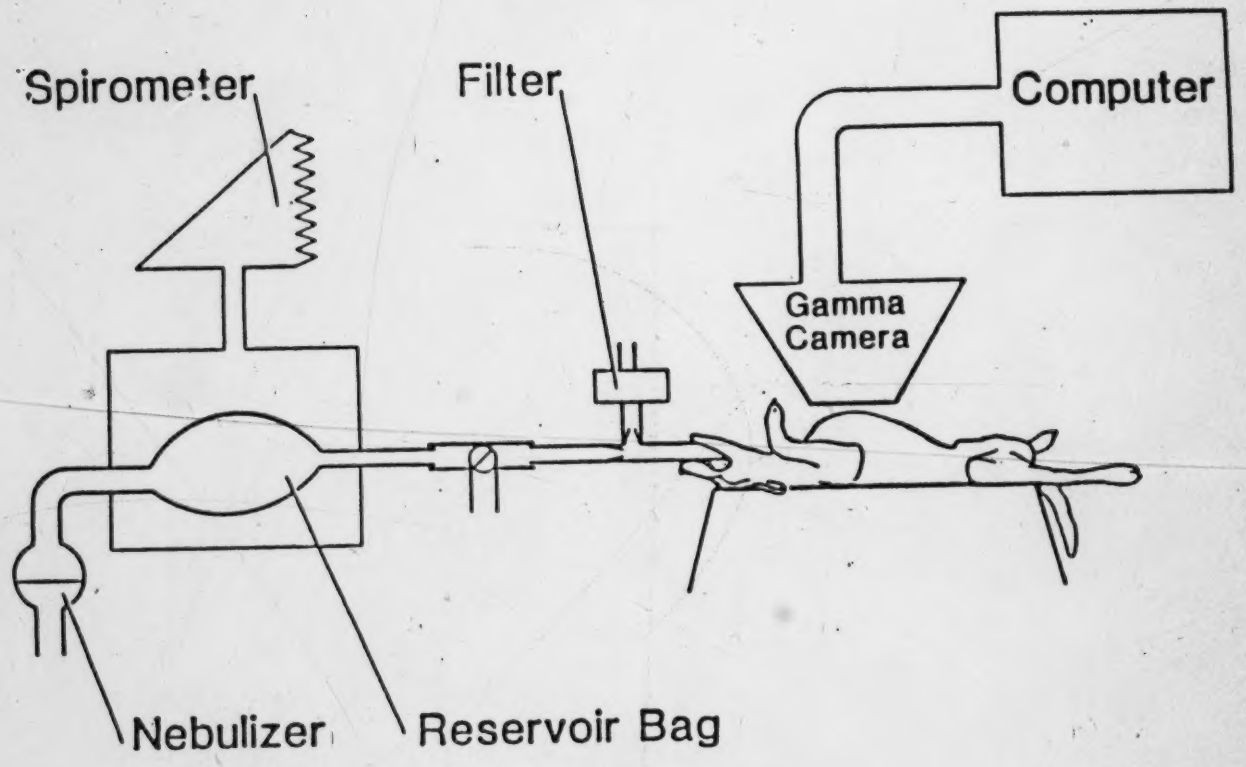


Figure IV: Experimental circuit for radioaerosol scanning. Dog breathing from aerosol-generating system of nebulizer and resevoir bag is positioned under gamma camera. Spirometer measured inspiratory excursions, and expired particles were trapped in filter.

Bronchoalveolar Lavage

The bronchopulmonary lavage procedure was the same for all animals and was adapted from that which was previously described for humans (Hunninghake et al 1979). An Olympus bronchoscope BF3 (Olympus Corp. of America, New Hyde Park, N.Y.) was inserted into the trachea via a number nine cuffed endotracheal tube and then advanced into the right bronchial tree. The tip of the bronchoscope was then wedged in a dependent subsegmental bronchus of the right middle lobe. Lavage was carried out by the instillation and immediate withdrawal of two 50-ml boluses of sterile 0.9% sodium chloride solution. The lavage took approximately 2 minutes from first instillation until last withdrawal. The segment of the middle lobe was chosen because its anterior location favors fluid recovery in the supine dog. Bronchoalveolar lavage fluid was pooled and strained through surgical gauze, and a fraction taken had total and differential count determined by staining (Papanicolaou stain) a cell pellet after spinning an aliquot in a cytocentrifuge. A second fraction was taken for albumin measurement and centrifuged at 900xg for 10 minutes at 4°C to remove cells and debris. The supernatant fraction and a sample of serum prepared from blood collected at the time of lavage were then assayed for albumin concentration (Sigma Procedure No. 631; Sigma Diagnostics, St. Louis, MO) with slight modifications; 10 μL was used for serum and 1 ml of bronchoalveolar lavage fluid instead of 10μL. The principle of the measurement depends on the fact that albumin binds to Sigma Diagnostics Albumin Reagent [BCG] which contains bromocresol green [BCG], to produce a blue green color with an absorbance maximum at 628 nm. The intensity of the color produced is directly proportional to albumin concentration in the sample. The concentration of albumin in each supernatant sample of BAL fluid and plasma was determined spectrophotometrically (Hewlett Packard 8451A Diode Array Spectrophotometer, Mississauga, Ontario) at 628 nm.

Static Pulmonary Compliance

This was measured using a modified technique (Muggenburg and Mauderly 1974, Zieler 1962). Changes in expired gas volume were measured with a dry gas volume spirometer (Ohio Medical Products, Houston, Texas). The transpulmonary pressure during expiration [pleural pressure (Ppl) - airway pressure (Pao)] was measured with a differential pressure transducer (Statham, Puerto Rico) and amplified with a Hewlett Packard recorder 7754B (Mississauga, Ontario). Airway pressure was sampled by a polyethylene tube (PE200 115 cm in length and 0.1575 cm internal diameter) attached to the side of the endotracheal tube. Pleural

pressure was estimated by positioning a compliant latex balloon (5.5 cm in length and 3.5 cm in circumference) on a catheter (PE200 80 cm in length and 0.1575 cm internal diameter) in the middle third of the esophagus. Prior to recording the pleural pressure, air was completely aspirated from the balloon and then 1 ml of air was re-introduced to standardize for the volume of air in the balloon. The final placement of the balloon was determined by locating a position in the esophagus that had minimal cardiac artifact as judged by there being no change in transpulmonary pressure when the dog inspired against a closed airway, and when there was a negative deflection in the transpulmonary pressure tracing on inspiration. This location was then fixed by taping the catheter to the endotracheal tube. The volume changes of the lung and transpulmonary pressure signals were recorded on an X-Y recorder (Hewlett Packard 7045A). Static pressure volume (PV) curves were obtained by first inflating the lungs with a syringe thrice, to obtain a transpulmonary pressure of +30 cm H_2O in order to establish a consistant volume history. The lung volume at +30 cm H_2O was assumed to be total lung capacity (TLC). After the last inflation, passive deflation from TLC to a volume below FRC was interrupted 4-5 times for 2-3 seconds each to give points in the PV relationship. At least 3 PV curves were obtained for each study. Static compliance (CL) in a given dog was measured over a volume from FRC (volume at which the alveolar pressure was zero) to 500 ml above FRC.

Functional Residual Capacity

FRC was measured with a helium dilution technique. A dry gas volume spirometer (Ohio Medical Products, Houston, Texas) was filled to a predetermined volume with a gas mixture containing approximately 10% helium in air. FRC measurement was preceded by 3 manual hyperinflations of 30 ml/kg to maintain the same volume history of the lungs and performed 2 minutes after the last hyperinflation. Helium gas concentration during rebreathing was monitored continuously with a helium analyzer (Ohio Medical Products, Houston, Texas). The volume of the lungs at FRC was calculated from the initial volume of the respiratory circuit, and the helium concentrations before and after the equilibration of the gas between the lungs and rebreathing apparatus. Values of FRC were corrected to BTPS. Determinations of FRC were done 3 times during each experiment and average values were reported.

Metabolic Function of Pulmonary Microcirculation

A polyethylene catheter (PE200) was advanced from the femoral artery into the thoracic aorta for measuring arterial pressure and sampling blood. Another similar catheter was passed into the right atrium via either an external jugular or femoral vein. Catheter position was confirmed by pressure tracing. The right atrial line was the site of injection, when radiolabeled substrates removal in vivo was determined with the single pass multiple indicator dilution technique (Gillis et al 1979). Briefly, after a bolus of about 1 ml containing an intravascular marker 2.5 mg of indocyanine green dye [Cardiogreen (ICG) Sigma, St. Louis, MO], in distilled water and a trace amount of ^{14}C-5HT (0.25 μmol) and ^{3}H-PGE$_1$ (0.1 μmol), was injected rapidly into the right atrium, aortic blood was sampled with a rotary pump (Gibson minipuls-2) at a rate of 60 ml/min, and directed into sampling tubes of a fraction collector (Pharmacia, Frac-100) at a rate of one sample/0.6 s, for a total of 30 samples. Radiolabeled compounds were obtained from Amersham, Ontario Canada; specific activity was 57 mCi/mmol for 5HT and 40.7 mCi/mmol for PGE$_1$. Each tube in the fraction collector contained 1 ml of chilled saline with one millimol of unlabeled 5 HT and 1 unit of heparin (Sigma Chemicals). Blood collection tubes were then centrifuged at 900 xg for 15 min at 4°C to separate the supernatant from the blood cells. ICG concentration in each supernatant sample was determined spectrophotometrically (Hewlett Packard 8451A Diode Array Spectrophotometer, Mississauga, Ontario) at 800nm. Radioactivity of ^{14}C-5HT and ^{3}H-PGE$_1$ in an aliquot of each supernatant was determined by a liquid scintillation counter (Beckman LS 7500 counter). The first 6 samples were collected before the injected materials had passed through the lung circulation. A known aliquot of the bolus injection solution was added to each of the first 3 blood samples collected which were processed in a similar fashion as above to estimate the total amount of ICG and radioactivity injected. The next 2 tubes were used for background activity calculation. The bolus volume injected was calculated from the weight of the syringe before and after the injection. Fractional concentrations based on the concentration of each substance normalized to the amount injected were plotted against the time of sampling.

Cardiac output and mean transit times were calculated as follows:

$$\text{Cardiac output} = \text{total ICG injected} \Big/ \int_{t_1}^{t_2} C_{icg(t)}\,dt \qquad (3)$$

$$\text{Mean transit time} = \text{appearance time} + \int_{t_1}^{t_2} tC_{icg(t)}\,dt \Big/ \int_{t_1}^{t_2} C_{icg(t)}\,dt \qquad (4)$$

where $C_{icg(t)}$ was the fractional concentration of ICG at time t, t_1 was the time at which ICG was first measured in the blood from the femoral artery, and t_2 was the time at which the logarithmic extrapolation of the ICG downslope until fractional concentration reached 2% of its peak value. Removal of the metabolic substrate was calculated as:

$$\text{Removal (t)} = 1 - C_{\text{Isotope(t)}}/C_{icg(t)} \qquad (5)$$

where C Isotope (t) is the fractional concentration of either ^{3}H-PGE_1 or ^{14}C-5HT at time t. Instantaneous removal at intervals around the peak of 5 HT and PGE_1 outflow curves was calculated.

Morphological Studies

Animals were sacrificed at different time intervals after NO_2 exposure for morphological studies. Of seven dogs sacrificed, one was sacrificed 1 hour after NO_2 exposure, two each were at day 2 and 7 post- NO_2 exposure, and one at 14 days post-NO_2. Four of the animals sacrificed were exposed to 200 ppm and two to 400 ppm NO_2. Of the control, one was sacrificed after compressed air exposure for one hour. The dogs were anesthetized with pentobarbital, systemically heparinized and killed by rapid exsanguination. A median sternotomy was performed and the heart and lungs were removed en bloc. The right lung was dissected free, degassed, infused with and placed in formalin (10% formalin at 30 cm H_20 of hydrostatic pressure. This fixation process was continued for 3-5 days. Each lower, middle and upper lobe was cut into five slices. Tissue samples from each slice were processed for light microscopy using standard techniques and stained with hematoxylin and eosin. The morphology of all tissue sections was interpreted by a pathologist blinded to the inhalation history.

Statistical Analysis

For statistical purposes each animal served as his or her own control. Differences between values were compared by analysis of variance and a p value of $p<0.05$ was accepted as being statistically significant. All data are presented as means +/- standard error unless otherwise specified.

9. RESULTS

Pulmonary Physiological Status

No changes in respiratory rate, tidal volume or minute ventilation were noted after compressed air inhalation. Immediately after NO_2 inhalation there was a 2-3 fold increase in the respiratory rate and a 2-3 fold decrease in tidal volume. There was a greater increase in respiratory rate with higher NO_2 concentrations (Tables 4,5).

There was a fall in the arterial 0_2 tension (PaO_2)(~ 15 mmHg) within 1 hour after 200 ppm NO_2 exposure, but saturation was above 95% following NO_2 inhalation irrespective of the concentration of NO_2. A trend towards a greater decrease in PO_2 following the higher concentration of NO_2 was observed (Table 5). Arterial CO_2 tension (PCO_2) values tended to be higher post-NO_2 exposure, suggesting less effective alveolar ventilation (Tables 4, 5).

FRC results are shown in Table 6. The pre and post compressed air values were highly reproducible. After NO_2 inhalation there was a fall in FRC within 1 hour after NO_2 exposure and at day 2 post NO_2. FRC values returned to normal by day 7 post NO_2 exposure. Collectively, the results indicate that there was a decrease in FRC after NO_2 exposure.

Static pulmonary compliance results are illustrated in Table 7. Irrespective of the concentration of NO_2, the shape of PV curves was the same before and after NO_2 exposure, and compliance measurements were unchanged.

Radioaerosol Scans

The dramatic increase in respiratory rate precluded radioaerosol measurements one hour after NO_2 exposure in 2 animals. In the remaining animals, visual assessment showed aerosol deposition to be peripheral, and no central activity was detected in the scans. In all animals except one which had an inadvertent intubation of one lung during the control scanning, little variability in the control values of $t_{1/2}$, the time it took for 50% clearance of the radiolabel from the region of interest (ROI), of $^{99m}TcO_4^-$ or ^{99m}Tc-DTPA was noted between right and left lungs.

Figure V shows the results of 7 dogs exposed to 200 ppm NO_2 for 1 hour. $T_{1/2}$ of

VENTILATION AND GAS EXCHANGE PARAMETERS EFFECTS OF 200 PPM NO_2

	Pre-Air	Post-Air	Post-NO_2		
			1 hr	2 days	7 days
n	(8)	(8)	(8)	(6)	(6)
PaO_2(mmHg)					
mean	80.1	79.1	63.3	70.7	77.7
SE	2.13	1.29	1.71	1.82	2.55
Pa CO_2(mmHg)					
mean	41.1	42.2	46.7	43.8	44.4
SE	1.45	1.25	2.4	2.06	1.55
pH					
mean	7.36	7.36	7.32	7.37	7.35
SE	0.01	0.01	0.03	0.01	0.01
Respiratory Rate per min.					
mean	11	12	28	20	11
SE	0.48	0.35	3.13	2.99	1.61
Tidal Volume (ml)					
mean	268.8	270.6	133.8	187.5	265
SE	30.72	28.5	11.17	18.70	32.12

Table 4 - A significant decrease in PaO_2 ($p < 0.05$) by anova occurred at 1 hr after NO_2 exposure. The fall in PO_2 was less at 2 days post-NO_2 and had returned to normal by 7 days post-NO_2 exposure. PCO_2 post-NO_2 had returned to normal by 2 days post-NO_2. A fall in pH was noted at 1 hr post-NO_2. Respiratory rate increased and tidal volume decreased significantly ($p < 0.05$) by anova at 1 hour and day 2 post-NO_2 exposure. All values returned to normal by 7 days post-NO_2.

VENTILATION AND GAS EXCHANGE PARAMETERS
EFFECTS OF 400 PPM NO_2

	Pre-Air	Post-Air	Post-NO_2 1 hr	Post-NO_2 2 days	Post-NO_2 7 days
n	(4)	(4)	(4)	(4)	(4)
PaO_2(mmHg)					
mean	81.3	80.5	54.8	62	77
SE	1.11	0.65	1.55	0.91	.091
Pa CO_2(mmHg)					
mean	38.8	40.3	48.5	46.5	44
SE	1.38	0.48	1.85	0.96	0.58
pH					
mean	7.35	7.35	7.27	7.33	7.35
SE	0.01	0.01	0.023	0.01	0.01
Respiratory Rate per min.					
mean	11	11	33	26	11
SE	0.48	0.41	0.95	0.5	0.48
Tidal Volume (ml)					
mean	195	197.5	97.5	112.5	200
SE	21.02	20.57	8.54	12.5	18.71

Table 5 - A significant decrease in PaO_2 and increase in $PaCO_2$ occurred at 1 hour post NO_2 ($p < 0.05$) by anova. This was accompanied by a fall in pH. By 2 days post-NO_2 both $PaCO_2$ and pH had almost returned to normal. PaO_2 values at day 2 though greater than 1 hour post-NO_2 values was still significantly decreased. Marked increases and decreases in Respiratory rate and tidal volume respectively were evident at 1 hour and day 2 post-NO_2. All values returned to normal by 7 days post-NO_2.

FUNCTIONAL RESIDUAL CAPACITY
EFFECTS OF NO_2, litres

Dog #	Weight kg.	NO_2 Conc. ppm	Pre-Air	Post Air	Post No_2 1 hour	2 days	7 days	14 days
1	13	400	0.830	0.836	0.736	0.678	0.734	0.727
2	17	200	1.063	1.084	0.858	0.963	1.082	1.061
3	23	400	1.076	1.094	0.918	0.729	1.072	1.023
4	17.5	200	1.043	1.002	0.891	0.889	1.028	....
N	4		4	4	4	4	4	3
Mean	17.6		1.003	1.004	0.851	0.815	0.979	0.937
SE	1.8		0.050	0.052	0.035	0.058	0.071	0.086

Table 6: A decline in FRC at 1 hour and 2 days post-NO_2 exposure occurred. A trend to a greater decrease in FRC persisted in the 2 dogs exposed to 400 ppm NO_2. By day 7 and beyond, FRC recovery was complete. Each value was the average of 3 measurements.

PULMONARY STATIC COMPLIANCE
EFFECTS OF NO_2,litres/cm H_2O

Dog #	Weight kg.	NO_2 Conc. ppm	Pre-Air	Post-Air litres	Post No_2 1 hour	2 days	7 days
5	20.5	200	0.0865	0.0829	0.0831	0.0860	0.1054
6	17.0	200	0.1267	0.1293	0.1159	0.1591	0.1786
7	16.0	200	0.1763	0.1630	0.1570	0.1678	0.1786
8	24.0	400	0.3116	0.3080	0.3630	0.3377	0.3526
N	4		4	4	4	4	4
Mean	19.4		0.1753	0.1708	0.1798	0.1878	0.1943
SE	1.8		0.050	0.052	0.035	0.058	0.071

Table 7: A decline in FRC at 1 hour and 2 days post-NO_2 exposure occurred. A trend to a greater decrease in FRC persisted in the 2 dogs exposed to 400 ppm NO_2. By day 7 and beyond, FRC recovery was complete. Each value was the average of 3 measurements.

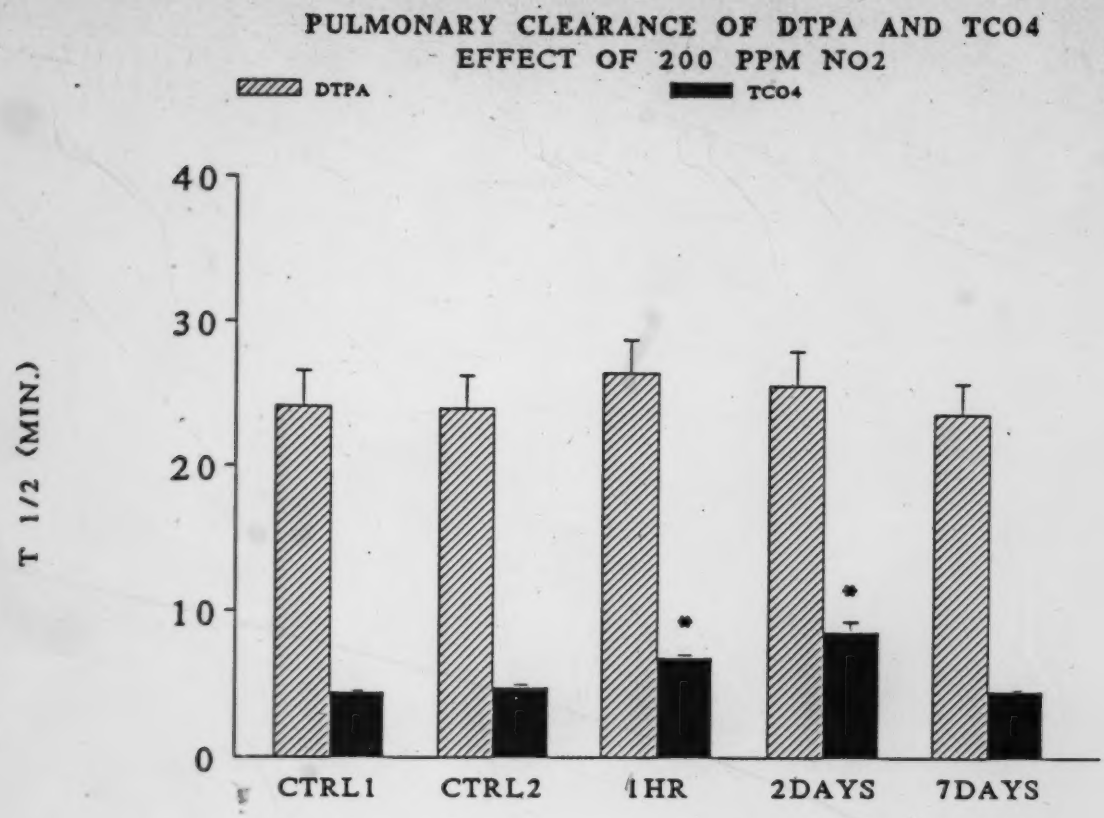


Figure V: * Significantly different from pre and post compressed air values (Ctrl 1,2)($p < 0.05$) by anova.
$^{99m}TcO_4^-$ and ^{99m}Tc-DTPA clearance ($t_{1/2}$) from right and left lungs of 7 dogs before and after NO_2. *Bars* +/- SE. $T_{1/2}$ was based on the first 30 min for $^{99m}TcO_4^-$ and the first 10 mins for ^{99m}Tc-DTPA. For ^{99m}Tc-DTPA clearance no significant changes were noted. $^{99m}TcO_4^-$ clearance showed a significant increase in $t_{1/2}$ at 1 hour and day 2 post-NO_2 exposure. One lung showed poor aerosol deposition in the control period due to inadvertent placement of endotracheal tube into opposite lung and this was excluded from the calculation. One dog was sacrificed after the second control measurement to serve as a control, and another one was not included in the post-NO_2 1 hour measurement because of rapid ventilation which persisted for hours after NO_2.

$^{99m}TcO_4^-$ clearance, based on the first 30 minutes after inhalation increased after exposure and reached a maximum value (≈ 186% of control) at day 2 post- NO_2. By day 7 post- NO_2 exposure, $t_{1/2}$ of $^{99m}TcO_4^-$ had returned to normal. On the other hand, $t_{1/2}$ of ^{99m}Tc-DTPA clearance did not change after NO_2, regardless of whether the calculation of $t_{1/2}$ of ^{99m}Tc-DTPA was based on the first 10 (shown in Figure V), 20 or 30 minutes after aerosol inhalation. Figure VI shows the results of 4 dogs exposed to 400 ppm NO_2. Both $t_{1/2}$ of $^{99m}TcO_4^-$ and $t_{1/2}$ of ^{99m}Tc-DTPA showed maximum increases (360% and 136% of control respectively) at day 2 post-NO_2 with a return to normal values by day 7. These results suggest that under the study conditions both concentrations of NO_2 altered the permeability of the alveolar capillary membrane with the greatest changes occurring at 1 hour and day 2 after exposure.

Bronchoalveolar Lavage Fluid

Examination of the airways at bronchoscopy after NO_2 exposure revealed mild erythema with a visible increase in secretions. NO_2 caused an appreciable alveolar -capillary leak as evidenced by a significant increase in lavage fluid albumin (Table 8). Although the total recovered volumes of lavage fluid were similar after compressed air and after NO_2 breathing, there was approximately a 460 percent increase in lavage-fluid albumin in response to NO_2. There was a significant increase in BAL total cell count post nitrogen-dioxide (Table 9). A significant increase in the percentage of polymorphonuclear leucocytes (PMNs) characteristic of acute inflammation and consistent with the histologic evidence of bronchiolitis and alveolitis described below was observed. The macrophage response to NO_2 exposure differed from that of the polymorphs. The percent of macrophages in lavage fluid showed a declining trend. However, macrophages were the major cell type in lavage fluids after compressed air or NO_2. By 14 days after NO_2 exposure BAL albumin concentration values had returned to control values. These results suggest that NO_2 altered the permeability of the alveolar epithelium at 1 hour after NO_2 exposure and that these effects were reversed after 14 days.

Hemodynamic Parameters

There were no significant changes in cardiac output or mean transit time for cardiogreen following NO_2 inhalation (Figure VII); the values were similar to those reported elsewhere (Muggenburg and Mauderly 1974). However, slight and variable changes were noted in the

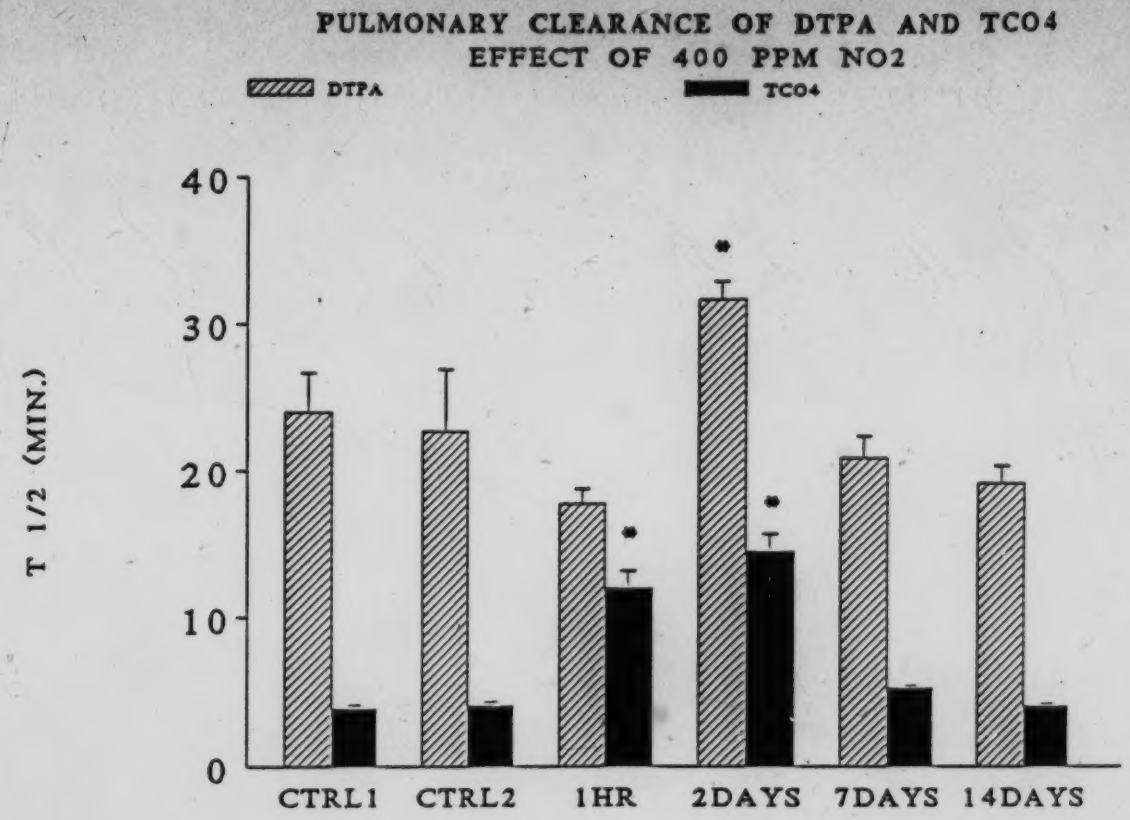


Figure VI: * Significantly different from pre and post compressed air values (Ctrl 1,2) ($p < 0.05$) by anova.
$^{99m}TcO_4^-$ and ^{99m}Tc-DTPA clearance ($t_{1/2}$) from right and left lungs of 4 dogs before and after 400 ppm NO_2. Error bars represent standard error of mean. Clearance of $^{99m}TcO_4^-$ decreased significantly at 1 hour and day 2 after NO_2. For ^{99m}Tc-DTPA clearance at day 2 was delayed. One dog had significant central airway deposition of aerosol and was not included in the early post-NO_2 study.

BAL ALBUMIN AND FLUID VOLUME EFFECTS OF NO_2

Dog #	BAL Volume (ml)		BAL Albumin (mg/ml)	
	Post-Air	Post-NO_2	Post-Air	Post-NO_2
14	67	67	.199	1.08
15	83	84	.16	0.5
16	63	64	.16	0.42
17	65	73	.18	1.25
18	70	65	.142	1.32
19	75	80	.155	1.16
n	6	6	6	6
mean	70.5	72.17	0.17	0.96
SE	2.77	3.10	0.01	0.15

Table 8: BAL Albumin and fluid volumes in 2 groups of three dogs each which received compressed air or 200 ppm NO_2. There was no difference in the volume recovered post-air or post-NO_2. Bal Albumin was significantly increased by NO_2 exposure, $p < 0.05$ by anova.

BAL CELL COUNT AND DIFFERENTIAL
EFFECTS OF 200 PPM NO_2

Dog #	Total Cells (10^4/ml)		Histiocytes %		Polymorphs %		Epithelial %	
	Post-Air	Post-NO_2	Post-Air	Post-NO_2	Post-Air	Post-NO_2	Post-Air	Post-NO_2
14	105	120	91	66	2	3	1	29
15	130	150	94	92	2	3	1	1
16	92	128	83	93	2	2	11	2
17	135	170	91	83	1	2	5	11
18	92	102	92	91	1	3	3	4
19	94	144	93	91	1	3	3	4
n	6	6	6	6	6	6	6	6
mean	108	135.7	90.7	84.7	1.5	2.83	4.17	9.83
SE	8.02	9.82	1.61	4.15	0.22	0.31	1.52	4.27

Table 9: BAL cell count (total and percent differential) post-air and at 1 hour post 200 ppm NO_2 exposure. A significant increase in total cells and percent polymorphs was noted post-NO_2 ($p < 0.05$) by anova. A trend towards a decrease in histiocytes percent and an increase in epithelial cell percent was observed post-NO_2. No change in percent lymphocyte count was seen. Each value was the average of four measurements.

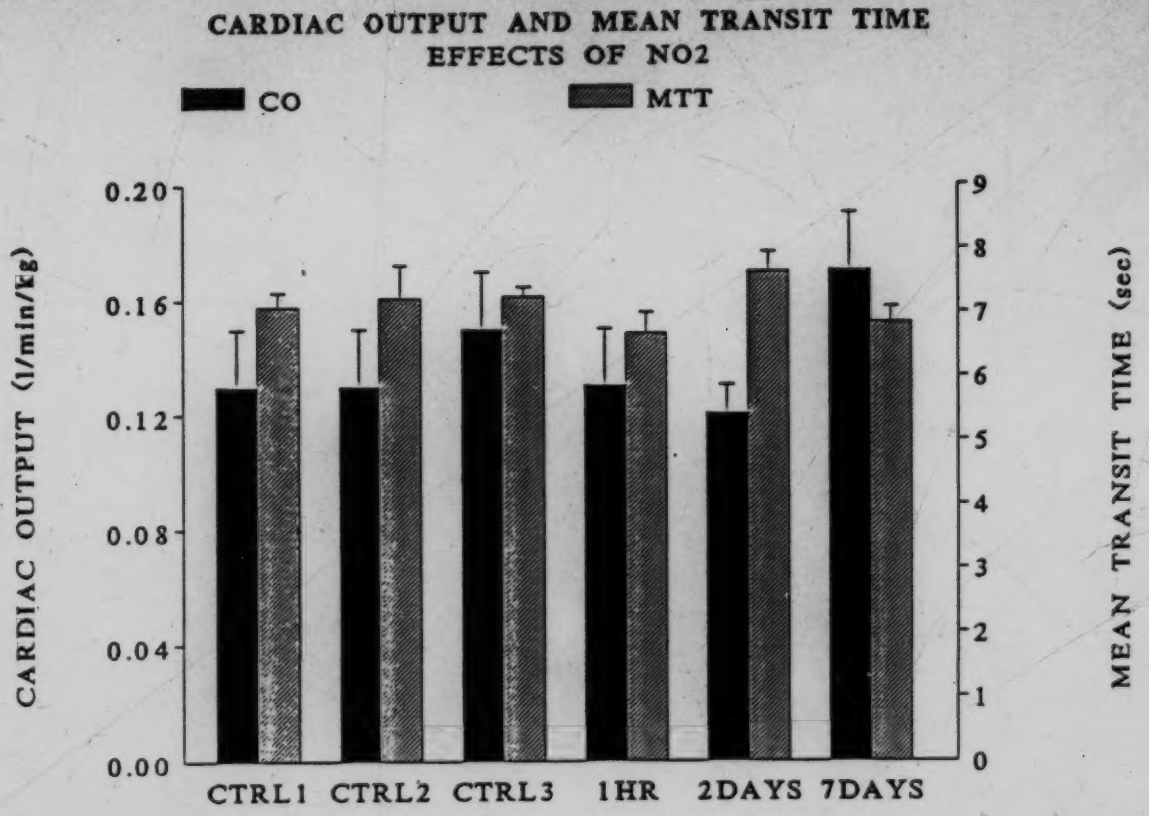
mean arterial blood pressure after NO_2 inhalation; some animals showed a slight decrease, other animals a slight increase in pressure. No direct or inverse correlations were noted between the concentration of NO_2 and mean arterial blood pressure changes.

Endothelial Function

A typical ^3H-PGE_1 and ^{14}C-5HT outflow concentration versus time curve and per cent removal (REM) obtained after compressed air is shown in Figure VIII. Following NO_2, the shape of the outflow curves were similar. The % removal (Equation 5) as a function of time indicated almost constant removal at time intervals around the peak before and after NO_2 for both 5HT and PGE_1. Figure IX shows a summary of the instantaneous % removal at the peak of the outflow curves. Pulmonary ^3H-PGE_1 and ^{14}C-5HT removal in the control period was 53.4% and 72.7% respectively. One hour after NO_2 exposure there was no significant change in the removal of ^3H-PGE_1 and ^{14}C-5HT (Figure IX). These results were also comparable to those at day 2 and day 7 or later on post-NO_2 exposure. The findings would indicate that NO_2 inhalation did not affect the single pass removal of ^3H-PGE_1 and ^{14}C-5HT when these compounds were given in tracer amounts.

Histological Studies

Plate 1 compares lungs exposed to NO_2 for 1 hour and the control. One hour after exposure to NO_2 there was acute bronchitis with focal but moderate interstitial and intra alveolar edema. However, these changes were patchy in distribution and minimal neutrophilic infiltration was present in the interstitium. By day 2, (Plate 2A) there was less edema, which was no longer evident by day 7 (Plate 2B). There was no evidence of atelectasis, fibrosis or emphysema in any of the sections at 7 or 14 days post NO_2. These findings are consistent with those of other workers (Dowell et al 1971, Evans et al 1972).



FigureVII: No significant changes were noted in the cardiac output or mean transit time measured by ICG in 8 dogs. In one dog in the control period and another at day 7 post-NO_2 technical problems prevented measurements. One dog was sacrificed after 1 hour exposure to NO_2 and two others 2 days after NO_2.

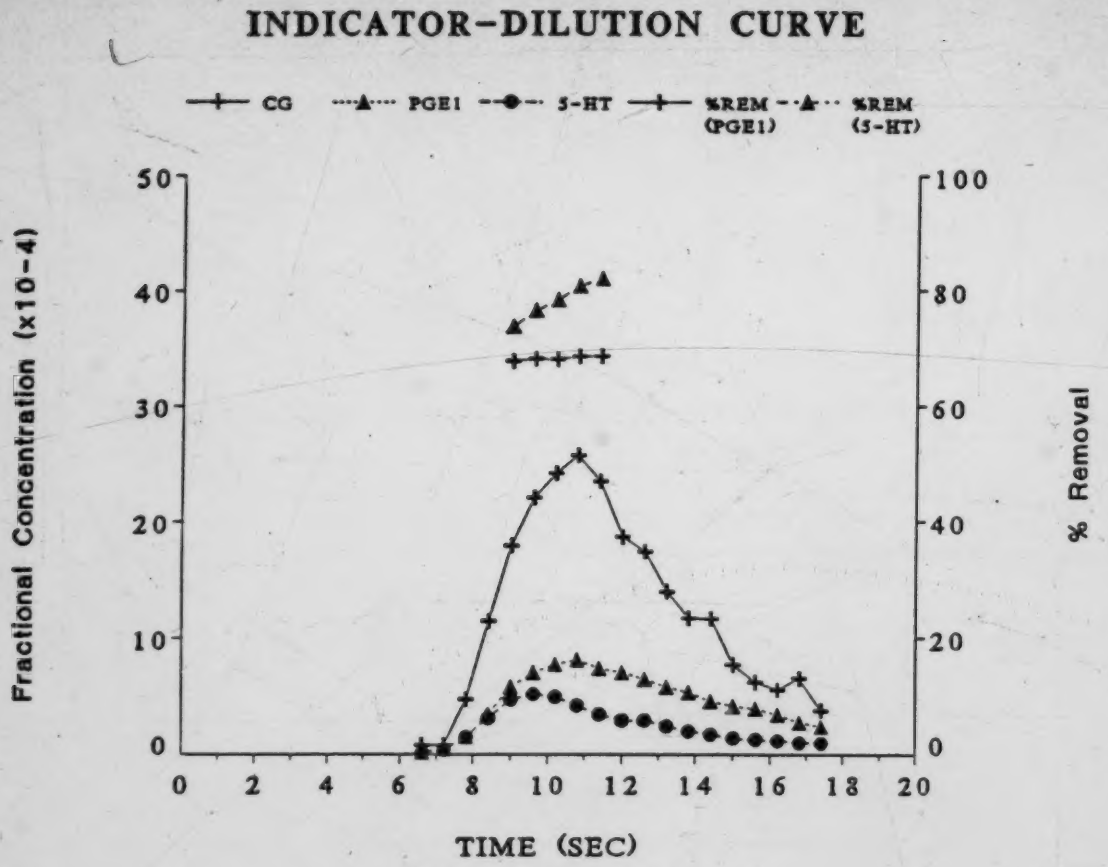


Figure VIII: Time concentration and per cent removal (REM) curves following injection of ^{3}H-PGE_1 and ^{14}C-5HT into the right atrium of one dog after compressed air. Relatively little difference was noted among any of the groups of animals at 1 hour, 2 and 7 days post-NO_2.

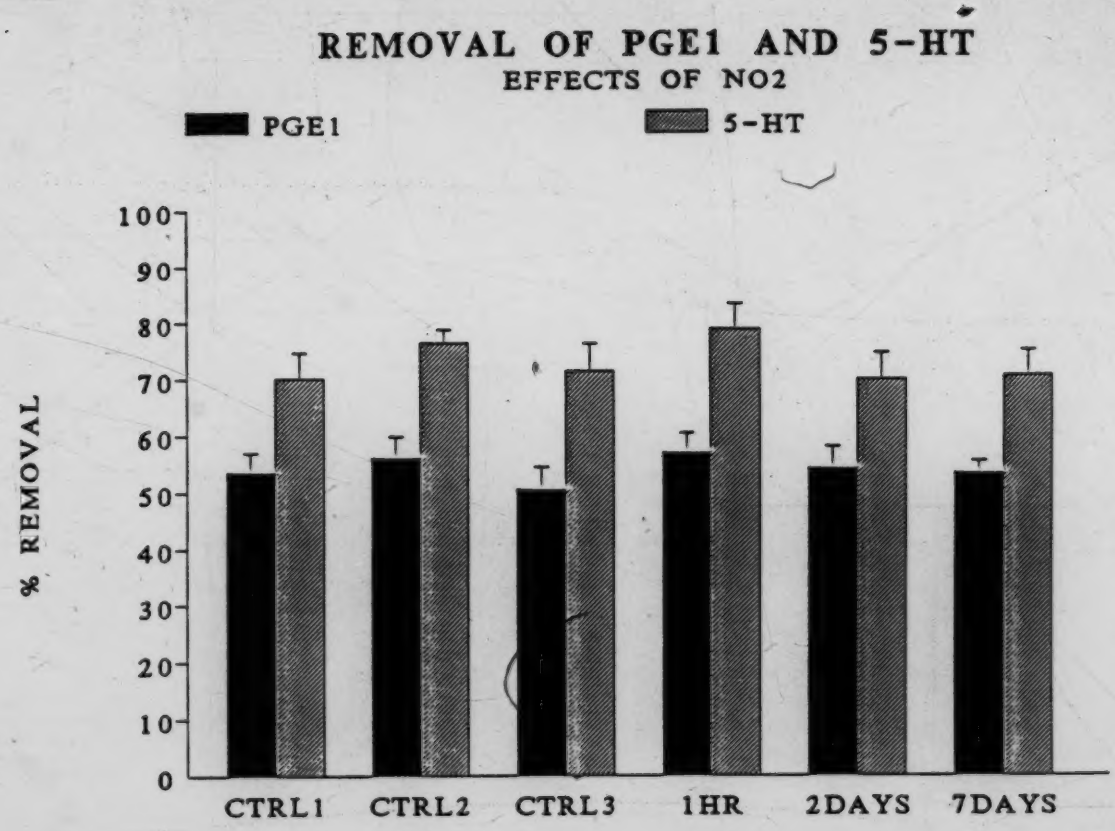
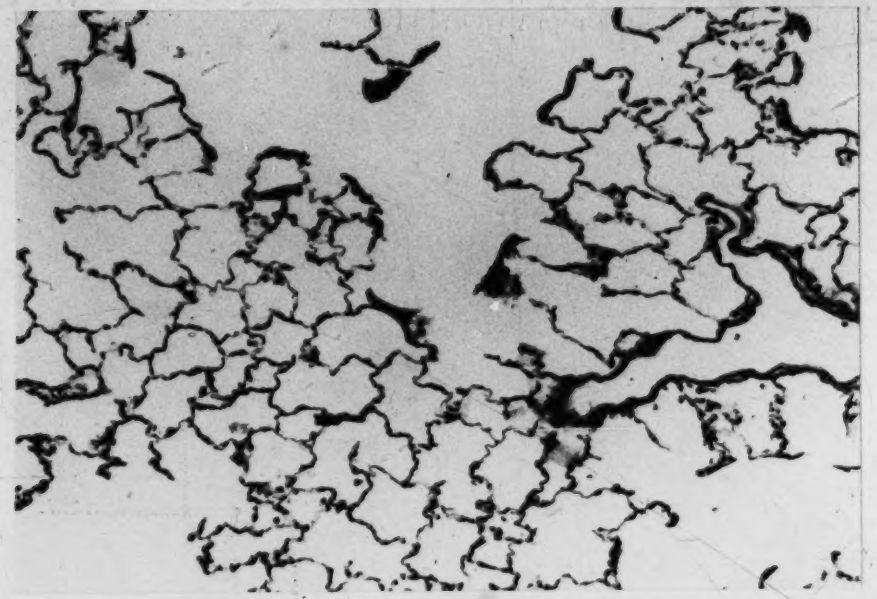


Figure IX: No significant changes were noted in the removal of PGE_1 or 5HT in 8 dogs. Technical difficulties precluded measurements in 2 dogs; one in the control period and another at day 7 post-NO_2. One dog was sacrificed after 1 hour exposure to NO_2 and two others 2 days after NO_2.

(A)



(B)

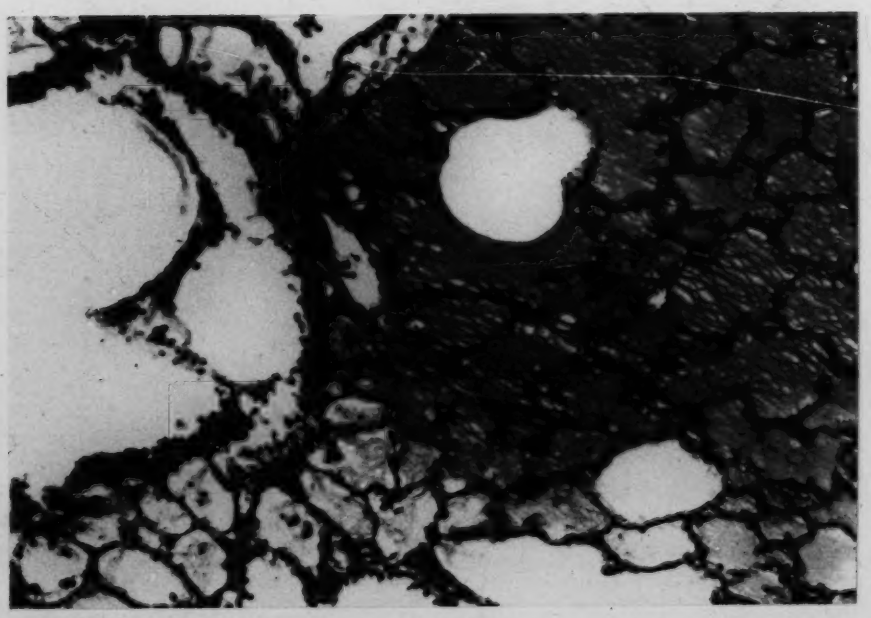
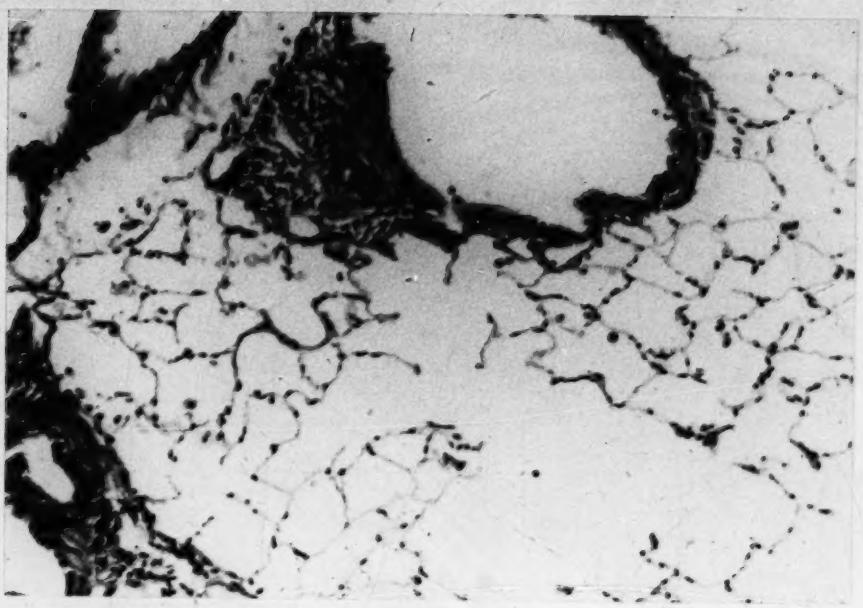


Plate 1 - Changes seen in light microscopy in samples taken from right lower zones of control (A) and 1 hour post NO_2 (B). A: normal histological appearance (x 160); B: intraalveolar exudation, and interstitial edema (x 160).

(A)



(B)

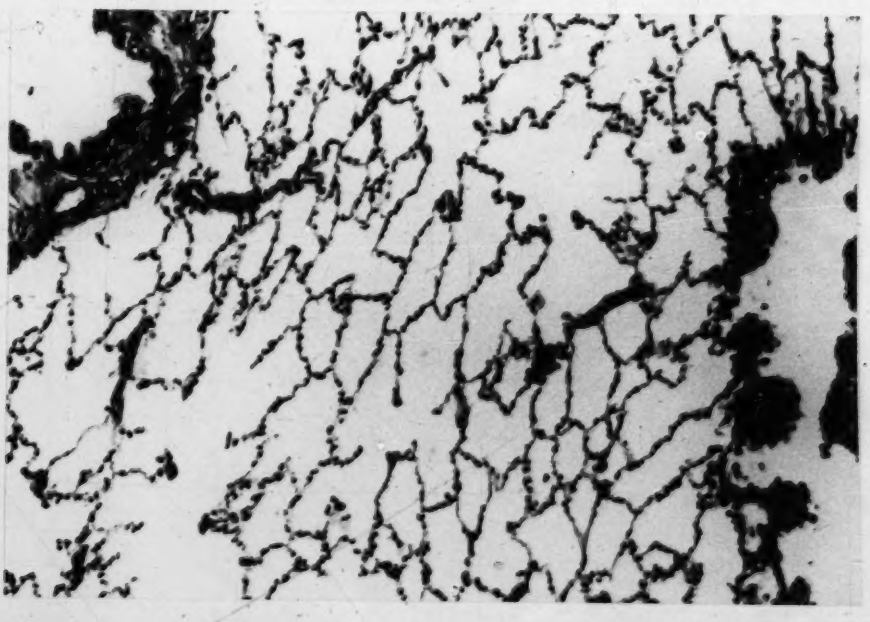


Plate 2 - Histological appearance of samples from right lower lobe of lung (A) 2 days post NO_2 and (B) 7 days post NO_2. Normal histological appearance in both.

10. DISCUSSION

The results demonstrated that high concentrations of NO_2 altered pulmonary clearance of $^{99m}TcO_4^-$ by 1 hour after NO_2 exposure, and that this alteration reached a maximum by the second day post-NO_2 exposure, irrespective of NO_2 concentration. For ^{99m}Tc-DTPA no significant change in clearance was noted after 200 ppm NO_2 exposure but a decrease in clearance was demonstrated 2 days after inhalation of 400 ppm NO_2. The $t_{½}$ of the clearance of both radioaerosols returned to normal by days 7 and 14 post-NO_2 respectively for 200 and 400 ppm NO_2. BAL albumin concentration increased by 1 hour after 200 ppm NO_2 exposure. These albumin changes occurred with visible tracheobronchial inflammation, were reversible and were associated with a small but significant change in the number and type of lung inflammatory cells in the lower respiratory tract. There was a simultaneous fall in the FRC as measured by the helium dilution method by one hour and this persisted until day 2. Due to the small number of subjects, no correlations between concentrations of NO_2 and changes in FRC were attempted. No changes in static lung compliance were noted. The lack of change in compliance was surprising, particularly in view of the trend towards a decrease in FRC. However, the number of animals used was small, the morphological changes were focal and patchy with the majority of lung sections showing normal histology with little or no involvement and it is possible that the technique used for compliance measurement was not sensitive enough. The observed initial response to NO_2 was focal, patchy acute inflammation of terminal bronchioles and adjacent air spaces, as well as interstitial and intraalveolar edema. The acute response subsided by 2 days and had returned to normal by 7 days. This histologic pattern of inflammation and edema was reflected in the changing composition of lung lavage fluid. The assessment of the metabolic function of the lung using ^{3}H-PGE_1 and ^{14}C-5HT, in contrast to radioaerosol clearance measurements, showed no significant changes at 1 hour, or days 2 and 7 post-NO_2.

Several authors have discussed in detail the limitations and interpretation of pulmonary epithelial permeability measurements using radioaerosol techniques (O'Brodovich and Coates 1987, Oberdoster et al 1986). A number of extraneous factors that could have affected the results such as the role of mucociliary clearance and changes in blood background are in general not significant causes for the marked alteration of $^{99m}TcO_4^-$ clearance. Transportation via the lymphatic system and by alveolar macrophages is also unlikely for the rate of clearance

via these mechanisms is much slower than the rates of clearance seen in the study. It is therefore likely that particles of $^{99m}TcO_4^-$ and ^{99m}Tc-DTPA leave the alveoli by crossing the alveolar-capillary membrane into the pulmonary circulation.

The mechanisms of clearance of $^{99m}TcO_4^-$ are unknown. Though it has been proposed to be by a passive mechanism from air to blood side (Ahmed et al 1986, Man et al 1985) some workers have demonstrated uptake by mucosal cells in the gut (Hays 1973). Thus it is quite possible that the increase in $t_{½}$ of $^{99m}TcO_4^-$ may be related to increased retention by one or another population of cells in the lungs. This appearance of radionuclide in the tissues of the lungs would diminish clearance rates. On the other hand it should be noted that $^{99m}TcO_4^-$ is normally secreted by the stomach, colon and salivary glands (Harper et al 1962). If similar secretion occurs in the airways, clearance of the radionuclide would thereby also diminish.

Another explanation for the decrease in $^{99m}TcO_4^-$ clearance is that the diffusion distance from the air to the blood side of the alveolar-capillary membrane and/or the surface area of transfer may have changed. Transfer of a substance across a membrane is inversely proportional to the length of the diffusion pathway and directly proportional to the surface area of transfer. If the length of the diffusion pathway is increased and/or the surface area of transfer decreased then the transfer of the solute is decreased. In this study histological sections have demonstrated the presence of interstitial edema, tissue edema and inflammatory cells infiltration, which increased in, at least, some parts of the lung parenchyma, the diffusion distance for the transfer of this solute. Moreover, it has been demonstrated in many studies that NO_2 causes not only an increase in the volume of capillary endothelial cells but also the Type I and Type II pulmonary epithelial cells (Guidotti 1980, Chang et al 1986).

The site of deposition of the radioaerosols was probably predominantly within the terminal respiratory units because micronic aerosols are predicted to deposit predominantly in the non-ciliated distal portion of the normal respiratory tract (Chamberlain et al 1983). Nevertheless, it could be argued that bronchospasm, increased airway wall edema and alveolar edema induced in these dogs could presumably change aerosol deposition both within and among the terminal respiratory units from peripheral to more central airways. No major changes in the site of deposition on radioaerosol scanning were seen. However, the major determinants of regional deposition of aerosols are particle size, rate and depth of ventilation and regional ventilation (Brain et al 1979). In this study, respiratory rate increased by greater than two fold after NO_2 exposure, and tidal volume decreased by 50%. This altered rate and depth of breathing can affect the site of deposition (Valberg et al 1982). These authors showed

that rapid-shallow ventilation (as seen in our animals) enhanced large-airway deposition in excised dog lungs. This was later confirmed by (Oberdorster et al 1986) who demonstrated that in vivo bronchial absorption of ^{99m}Tc-DTPA was slower than alveolar absorption. Thus it is quite possible that preferential deposition of $^{99m}TcO_4^-$ in the peripheral airways which have thicker epithelium than alveoli could have resulted in a decreased clearance in $^{99m}TcO_4^-$. Moreover, the higher breathing frequency and shallower ventilation in the 400 ppm NO_2-exposed animals compared to the 200 ppm NO_2-exposed animals are associated with a greater increase in $t_{½}$ of $^{99m}TcO_4^-$ clearance at 1 hour and 2 days post NO_2 and also an increase in $t_{½}$ of ^{99m}Tc-DTPA clearance post 400 ppm NO_2. This decrease in clearance may thus be a result of more bronchial-airway rather than alveolar deposition of the radioaerosols. An assessment of how much change in bronchial versus alveolar deposition occurred was not made. These altered ventilation parameters however, do not fully explain the clearance of ^{99m}Tc-DTPA. No change in clearance was noted after 200 ppm NO_2. If, as suggested above, the site of deposition was the major determinant in the clearance of these radioaerosols, then like $^{99m}TcO_4^-$, the $T_{½}$ of ^{99m}Tc-DTPA after 200 ppm NO_2 should increase post NO_2. This did not occur, therefore other factors must be involved.

There was a trend that the lungs were somewhat smaller as evidenced by the reduction in FRC in those animals studied. The relationship between FRC and surface area of transfer is far from being clear, as the lung is not a perfect sphere. However, it can be safely assumed that if FRC is smaller, then surface area of transfer for $^{99m}TcO_4^-$ is not likely to become bigger. Although there was a decrease in FRC and an increase in $T_{½}$ of $^{99m}TcO_4^-$ post NO_2 a statistically significant correlation between these 2 variables could not be demonstrated. This may be due to a variety of factors including the small sample size, inherent variability in the measurements and the time lag of 30 min between FRC and $^{99m}TcO_4^-$ clearance measurements.

Marks et al (1985) have shown that in normal subjects breathing at different lung volumes induced by different levels of CPAP, that clearance was increased in an exponential manner as FRC was increased from 35 to 85% TLC. On the other hand, Meignan et al (1985) evaluated lung clearance of ^{99m}Tc-DTPA and the effect of postural changes. They found that in the lateral decubitus position clearance from the dependent lung (with smaller alveolar volume) was markedly slower than from the non-dependent uppermost lung (with larger alveolar volume). Other workers (Rinderknecht et al 1980) have found that dependent and non-dependent lung regions have the lower and higher clearances respectively. Nevertheless,

because these few animals showed decreased lung clearance of $^{99m}TcO_4^-$ post-NO_2, the decrease in FRC suggests that NO_2-induced changes in permeability and pulmonary function may be related. Accordingly, therefore the reduction in $^{99m}TcO_4^-$ clearance could be due to a reduction in the surface area of transfer for $^{99m}TcO_4^-$ and/or an increase in the length of the diffusion pathway. These changes are not unique, and have been previously demonstrated in canine lungs after radiation treatment (Ahmed et al 1986).

The role of intra-alveolar edema with particular reference to protein and lipid in the alveoli, and their relationship to clearance of radioaerosol is unknown. Albumin is known to bind $^{99m}TcO_4^-$ (Hays and Green 1973). It has been demonstrated that in pulmonary alveolar proteinosis, a condition characterized by the deposition within the airspaces of the lung of a granular material high in protein and lipid content, that clearance of $^{99m}TcO_4^-$ is decreased (Rinderknecht et al 1980). It has also been postulated that this precipitated material composed of modified surfactant and serum reduces access of the radionuclide to the alveolar surfaces and thereby decreases solute clearance; for removal of this proteinaeous material by lavage significantly increased radionuclide clearance. Therefore the binding of $^{99m}Tc0_4^-$ to proteins could explain the slower clearance of this solute across the alveolar-capillary membrane (Rinderknecht et al 1980).

With respect to lipid material in the alveoli; NO_2 exposure in well known to alter lung phospholipid and total lipid composition (Williams et al 1971, Blank et al 1978). For example, lipid epoxide formation is increased in triglyceride, cholesterol, and phospholipid fractions derived from the lungs of rats exposed to NO_2 (Sevanian 1979). In addition NO_2 exposure resulted in an increase in the ratio of saturated species of phospholipids to unsaturated species of phospholipids in rat and hamster lungs (Willliams et al 1971, Blank et al 1978). Both $^{99m}TcO_4^-$ and ^{99m}Tc-DTPA are lipophobic, hydrophilic solutes so an increase in membrane-saturated lipids in the alveoli would be expected to decrease translocation of these solutes across the alveolar epithelium. Moreover, it has been revealed that surfactant depletion in rabbits by lung lavage which removes alveolar phospholipid results in an increased clearance of ^{99m}Tc-DTPA; and if exogenous natural surfactant is then placed in the alveoli, clearance of the radioaerosol is faster than controls but slower compared to that of the surfactant depleted lungs (Wollmer et al 1986).

In the present study morphology revealed intra-alveolar edema and bronchoalveolar lavage demonstrated an increased albumin concentration in the lower respiratory tract after NO_2 exposure. It is thus possible that the altered volume and content of the epithelial lining fluid

could diminish access of the radionuclide to the alveolar surface and so result in a slower clearance of $^{99m}TcO_4^-$.

The interpretation of the clearance of ^{99m}Tc-DTPA might be as follows: at present no known active mechanism for epithelial translocation has been described for this solute, but because of its size, it is generally believed that this solute moves across the epithelium through the paracellular pathway. As well, it is possible that this solute may be weakly linked to proteins present in the alveolar fluid, thereby further limiting its transfer by the transcellular route. For ^{99m}Tc-DTPA it is clear that in any two compartment system, the half time for equilibration across the transport barrier will depend upon the barrier permeability, exchange area, and the volumes of both compartments; as is true for all other solutes used in radioaerosol studies. The surface area of transfer is different for $^{99m}TcO_4^-$ and ^{99m}Tc-DTPA and in this case, following 200 ppm NO_2, there was no significant change in the clearance of ^{99m}Tc-DTPA, but there was a marked decrease in the clearance of $^{99m}TcO_4^-$. Moreover, in this study the volume of the fluid in the alveolar space into which the isotope dissolved was increased as revealed by histology. Thus, it would be expected that even if permeability was constant an increase in alveolar fluid volume would at least cause the half time clearance of ^{99m}Tc-DTPA to increase. In fact no significant change occurred with 200 ppm NO_2 which is in agreement with the study of Oberdorster et al (1986) that used 5 ppm NO_2 in rabbits. This could therefore imply either that the alveolar epithelial permeability was not increased (in contradistinction to the documented increase in BAL albumin concentration) and/or that the relative exchange area in non fluid-filled ventilated alveoli for transfer of ^{99m}Tc-DTPA was increased compared to that of fluid-filled alveoli where a marked decrease in clearance would be expected. The latter suggestion would indirectly imply an enlargement of the paracellular pathway in the non fluid-filled ventilated alveoli. Indeed, this approach has been used successfully to understand epithelial permeability changes in a variety of experimental conditions, such as radiation lung injury (Ahmed et al 1986), and effects of positive end-expiratory pressure and high frequency ventilation on pulmonary permeability (Man et al 1987). Furthermore, it has been reported that a complete restoration of normal fibril number and organization occurred 2 days after 30 ppm NO_2 exposure for 4 hours (Case et al 1983). This would be in keeping with the fact that in this study the ^{99m}Tc-DTPA clearance did not change at 2 days post NO_2 after 200 ppm NO_2, implying that paracellular permeability, a reflection of tight junction integrity was restored to normal. Interestingly similar results of decreased alveolar clearance of ^{99m}Tc-DTPA was demonstrated in patients with primary ciliary

dyskinesia. This decreased clearance has been postulated to be related to a supernormal lining of the alveoli by surfactant factor (Groth et al 1986).

It is by no means clear what a change in FRC would do to the clearance of ^{99m}Tc-DTPA. Nor is it obvious why a change in FRC would have a greater effect upon the t½ of $^{99m}TcO_4^-$ than ^{99m}Tc-DTPA. The explanation may lie in their different molecular weights and possibly different pathways for translocation. However, the results in this study are in partial agreement with those of Oberdorster et al (1986) who showed that in rabbits after exposure to 5 ppm NO_2 at normal tidal volumes the clearance of ^{99m}Tc-DTPA did not change. However, when tidal volumes were increased the clearance of ^{99m}Tc-DTPA was decreased. This is in contrast to what is expected and suggests that in NO_2-induced lung injury factors other than lung volume play an important part in permeability of these radioaerosols.

This study demonstrated that NO_2 in high concentrations caused an increase in the concentration of albumin in BAL fluid, a finding suggesting that permeability of the lung epithelium is increased. The concentration of albumin in BAL fluid, however, can be influenced by the technique of lavage (Merrill et al 1982), processing of the fluid (Halliwell 1984), and the presence of lung disease (Dusser et al 1984). In this study, uniformity of procedures and the normality of the dogs limited these effects. A volume equal to or greater than 100 ml has been suggested as being sufficient to sample lung proteins accurately (Merrill et al 1982). The volume of BAL fluid return was not different after compressed air or NO_2 and therefore could not explain the albumin concentration differences seen.

In contrast to BAL albumin concentration, ^{99m}Tc-DTPA clearance was unchanged after exposure to 200 ppm NO_2. This was a surprising finding in light of the smaller size of ^{99m}Tc-DTPA (492 daltons) as compared with albumin (69,000 daltons), which would predict a greater sensitivity of ^{99m}Tc-DTPA clearance to changes in membrane structure and function. Furthermore $^{99m}TcO_4^-$ of even smaller molecular weight (163 daltons) than albumin showed a decreased clearance across the alveolar-capillary membrane. No investigations have been done correlating radioaerosol clearance and BAL albumin concentration in NO_2 inhalational lung injury. However, some workers have reported differences between ^{99m}Tc-DTPA clearance and BAL albumin concentration changes in human studies. Clearance of ^{99m}Tc-DTPA is increased in smokers (Mason et al 1983) whereas there is no difference between smokers and nonsmokers in BAL albumin concentration (Bell et al 1981). Other investigators have shown that albumin can be a scavenger of toxic oxygen radicals (Holt et al 1984) so that increased albumin flux across the alveolar-capillary membrane and airway epithelium might protect

against the early changes of NO_2 toxicity. Differences in sensitivity between the 2 methods suggest that they are measuring different processes involved in solute movement. Possible active transport of albumin in the alveolar to pleural direction occurs in bullfrog lung epithelium (Kim et al 1985) and from the albuminal to luminal side of pulmonary endothelial cell monolayers in culture (Shasby and Shasby 1985). Blockade of albumin active transport by NO_2 at either site might lead to accumulation of albumin in the lung. It is tempting to speculate that the changes in BAL albumin concentration and clearance of $^{99m}TcO_4^-$ and ^{99m}Tc-DTPA may be indicative of cell damage and NO_2 toxicity. Although biochemical evidence of cell injury in the BAL fluid was not evaluated, lung inflammatory cells and epithelial cells did change significantly, the changes were rapidly reversible however, suggesting that lung cell damage, if present at all, was minimal. The findings are still compatible with early NO_2 toxicity because permeability changes can occur in the absence of morphologic evidence of lung injury (Guidotti 1980).

The mechanism of the NO_2 effect on the alveolar capillary membrane and airway epithelium is unknown, but one popular theory is that NO_2 directly injures lung parenchymal cells through formation of toxic oxygen radicals. These free radicals may cause lipid peroxidation of the tight junction areas of the cell wall, thus leading to loss of barrier function (Mustafa and Tierney 1978). Disruption of tight junctions is associated with increased epithelial permeability to macromolecules (Crandall et al 1983, Schneeberger and Lynch 1984) and cell membrane peroxidation is an early effect of NO_2 (Patel and Block 1986). A toxic effect of NO_2 on the cell membrane is supported by experiments showing a loss of cell membrane metabolic activities and decreased membrane fluidity of cultured endothelial cells exposed to NO_2 (Patel and Block 1986). NO_2 has been shown to disrupt lung epithelial tight junctions (Case et al 1983, Gordon et al 1986). This provides further evidence that NO_2 alters the barrier function of the alveolar epithelium.

An alternative theory to the "direct toxic hypothesis" of NO_2 is that much of the damage induced by NO_2 is caused by inflammatory cells, probably neutrophils that have been attracted to the lower respiratory tract either directly or indirectly by the action of the high concentrations of NO_2. Morphologic studies of dogs exposed to NO_2 show neutrophils in the interstitium (Guidotti 1978). Although there is no question that inflammatory cells may have a role at some stage in NO_2-induced disease, there is clear evidence that NO_2 can injure lower-respiratory-tract parenchymal cells without the influx of inflammatory cells. For

example, morphologic studies of dogs (Guidotti 1980) clearly showed endothelial and epithelial cell damage before any accumulation of inflammatory cells. Nevertheless, acute NO_2 inhalation is associated with increase numbers of inflammatory cells and it remains possible that histiocytes, PMNs and cells in the interstitium may play a very important role in the metabolism and permeability of the alveolar capillary membrane. Therefore, their contribution to permeability and metabolism cannot easily be dismissed.

Few studies have compared pulmonary clearance of radioaerosols after brief exposure to high concentrations of toxic gases. However, in this laboratory the effects of a single exposure of high concentrations (100 ppm and 500 ppm) of sulfur dioxide (SO_2) on pulmonary epithelium in adult dogs over a period of several weeks have been evaluated (Man et al 1988). We found that in vivo radioaerosol clearance using ^{99m}Tc-DTPA and $^{99m}TcO_4^-$ did not change after exposure to 100 ppm and 500 ppm SO_2, but mucociliary transport rates did. We concluded that the solubility of SO_2, the scrubbing effect of the upper airways together with a more severe damaging effect of SO_2 specific on the ciliated cells might be possible explanations for the absence of change in radioaerosol clearance.

While pulmonary permeability changes have not been examined with a range of different concentrations of NO_2, it was evident that in comparison to 200 ppm NO_2, 400 ppm NO_2 appeared to cause more persistent changes in pulmonary epithelial permeability which required a longer recovery period. That normal histology and alveolar permeability returned with time is in keeping with earlier morphological findings (Evans et al 1972, Stephens et al 1972) which suggest that with NO_2 injury, epithelial damage is predominant within the first 24 to 48 hours and returns to normal by seven days.

Multiple mechanisms could therefore be responsible for the NO_2-induced alteration in respiratory epithelial permeability. The injury to ciliated airway epithelium and type I alveolar cells that was observed in animals after acute exposures to 30 ppm NO_2 (Guidotti et al 1980, Bito 1974) and the interstitial and alveolar edema observed in the present study provide a potential morphologic correlate for the alteration in epithelial function. It is unlikely that aerosol particles reached any fluid-filled alveoli. Therefore, it is reasonable to suggest that clearance is occurring from non fluid-filled or less damaged alveoli and terminal or respiratory bronchioles. It is thus conceivable that these non fluid-filled alveoli are receiving more radioaerosol than other fluid-filled alveoli and areas where increased resistance due to edema and mucus such as the terminal bronchioles occur. These former regions would therefore have possibly faster clearances than the latter areas. Thus this effect of regional differences in

aerosol deposition could significantly alter regional removal of radioaerosol from the lungs. Therefore differences could be influenced by both the concentration gradient across the epithelium and the site of aerosol deposition. The regional concentration gradient cannot easily be known. The aerosol concentration in the fluid layer on the alveolar surface is indeed determined by the amount of aerosol deposited and the alveolar surface area reached by the particles. The size of the surface area depends on the alveolar volume and the density and shape of the alveoli, all parameters which are not accurately known.

It was of importance to note that despite the changes in the aerosol studies there was no documented depression in ^{3}H-PGE_1 or ^{15}C-5HT removal. The interpretation of these measurements depends on understanding the role of heterogeneity of blood flow, cardiac output, mean transit time and others, and these have been expertly reviewed (Goresky et al 1970). Several reasons may be advanced as plausible explanations for the findings. First, endothelial cells were not damaged, and NO_2 produced mainly epithelial cell dysfunction. This is not very likely because although the presence of edema in the histological sections, and increased BAL albumin concentration would indicate at least an alteration of the barrier function of the alveolar epithelium; NO_2 exposure is known to cause injury to mammalian pulmonary endothelial cells in vivo (Brown et al 1983, Guidotti 1978). In addition, the diffusion of inhaled NO_2 into the blood through the endothelium and the appearance of NO_2 at extra-pulmonary sites in rhesus monkeys (Goldstein et al 1977) suggests that pulmonary endothelial cells should have been in contact with NO_2. Nevertheless, it has been demonstrated that uptake of 5HT by cultured endothelial cells is not depressed after 3 or 12 hours exposure to 5 ppm NO_2 but only after 24 hours (Patel and Block 1986).

Consideration must also be given to the possible influence of systemic and pulmonary hemodynamic fluctuations in the estimations of endothelial metabolic measurements. Such changes may or may not affect removal values. One variable that is not adequately controlled yet, is the estimation of mean transit time through the "exchange" or metabolically active microvessels and of changes therein. Although indicator transit times were measured between the site of injection (right atrium) and the site of withdrawal (thoracic aorta) this provides little information about possible changes in capillary transit times. Estimations of transit times through the pulmonary capillaries in man have yielded average values of $\sim$ 0.8 sec, this is ~ 7-8 times less than the dog mean pulmonary transit times in this study. Thus the method used in this investigation may be unable to detect even great alterations in true capillary transit time. The fact that mean transit times did not change post-NO_2 may thus indicate either that there is no significant heterogeneity in transit times through the pulmonary microvessels under the

conditions of this study or that such heterogeneity exists but was not detectable. Nevertheless, it is conceivable that cellular swelling, intraalveolar and interstitial edema all documented with NO_2 inhalation might change blood flow in the microcirculatory vessels and so alter heterogeneity in capillary transit times. However, no direct evidence to suggest that is available for this study.

Despite the injury, it is possible as has been noted for other pulmonary metabolic functions (Ryan et al 1982) that the number of carrier sites may be in great excess of the circulating and exogenous 5HT or PGE_1, in this study. This may explain why the removal of these substrates was not depressed by high concentrations of NO_2, as the concentration of removal sites would be far in excess of the substrates. If more extensive damage had been caused, a depression in the percent removal might have been seen. In fact, it has been suggested that the removal of metabolic substrates is often limited by the rate of supply, rather than the rate of endothelial cell transport (Goresky et al 1970). Furthermore, as only trace amounts of these substrates were used, advantage of the fact that both 5HT and PGE_1 uptake are dependent on saturable processes could not be utilized to analyze the data according to Michealis Menten kinetics (Bito et al 1977, Linehan and Dawson 1979). Thus, Vmax (maximum uptake rate) or Km (substance concentration at Vmax/2) could not be defined although they might have indicated a significant change in certain properties of the enzyme systems after NO_2. Unfortunately, use of higher saturating concentrations of 5HT or PGE_1 (Rinderknecht et al 1981, Hyman et al 1978) is accompanied by pronounced hemodynamic changes which, per se, may affect quantitative estimates of removal. The indicator dilution method requires that blood volume and flow be constant during the bolus transit through the lung. Therefore, because estimates of substrates removal made in vivo with concentrations approaching their Km are very sensitive to small changes in pulmonary volume, pulmonary hemodynamic changes (e.g. vasoconstriction) will significantly change the concentration of the substrate in the lung microvasculature. This would result in a difference in the shape of the curve as compared to those obtained with trace amounts of substrates.

On the other hand, a loss of microvascular metabolic capacity could still have occurred. This could have been secondary to a decrease in surface area caused by microembolism, reflex vasoconstriction, pulmonary edema or unknown causes. The result of the pathology would be a preferential redistribution of blood flow to areas with intact endothelium. After NO_2 inhalation and the subsequent initial damage dormant and unexposed capillaries could have been recruited, hence a normal value for ^{14}C-5HT and ^{3}H-PGE_1 removal. Thus, it is possible that areas of damaged endothelium with reduced substrate removal could exist but because they were underperfused a certain degree of dysfunction could not be demonstrated. However,

there is at present no functional measurement of surface area that could be used in vivo. A recent report by Ryerson and coworkers (1981) attempted to quantify the effect of decreased vascular surface area on 5HT uptake, using elastase-induced emphysema in the hamster. They found no effect on 5HT disposition despite a marked diminuition in surface area. The failure to demonstrate depressed 5HT uptake was thought to be due to the great reserve capacity of the lung vasculature for removal of biogenic amines. Similarly, Flink and associates (1982) demonstrated that a significant portion of the vasculature must be obstructed, perhaps greater than 70% before a decline in removal of 5HT would occur. Moreover, in the presence of pulmonary edema a lack of effect on serotonin uptake by dog lungs was noted after oleic acid-, alpha-naphthylthiourea- and phorbol myristate acetate-induced microvascular damage (Gardaz et al 1988). These workers concluded that dissociation exists between metabolic dysfunction of pulmonary endothelial cells and fluid leakage. Therefore, widespread dysfunction must be present before the reserve capacity of the pulmonary endothelium is overwhelmed and cannot maintain normal function in early injury.

The mode of onset of lung damage, on the other hand, may be an important factor in the elicitation of defective pulmonary removal of ^{14}C-5HT and ^{3}H-PGE$_1$. The models used successfully for the demonstration of pulmonary endothelial damage from the measurement of biogenic amines and prostaglandins are all of rather slow or progressive nature. The time lag between administration of the toxic agent or the exposure to the toxic condition and demonstration of depressed substrate removal is usually greater than 12 hours; 12 hour hyperoxia (Dobuler et al 1982), 36 and 48 hrs hyperoxia (Klein et al 1978) and 24 hours NO_2 (Patel and Block 1986). In contrast, the development of pulmonary edema may take place within 1 hr of NO_2 exposure (as demonstrated in this study). Permeability alterations are thus probably partly associated with abnormalities at the levels of the intercellular junction, whereas decreases in serotonin and prostaglandin removal probably result from a more general metabolic dysfunction of the endothelial cells.

Another explanation centers around the use of ICG as the inert vascular tracer. It has been reported that ICG inhibited uptake of ^{3}H-PGE$_1$ in isolated dog lungs perfused with Krebs' solution, but the dye was relatively ineffective when similar experiments were performed in plasma perfused lungs (Dawson et al 1983). Thus it is possible that ICG inhibited the uptake of ^{3}H-PGE$_1$ and is responsible for the reduced removal of this substrate and so the absence of any significant decrease in removal of ^{3}H-PGE$_1$ after NO_2 exposure. While it is not possible to elucidate the exact reasons for the lack of endothelial metabolic changes, all the above might well have contributed to maintaining the seemingly normal removal of these substrates. Finally, technical considerations prevented correction for the

presence of radiolabeled metabolites derived from 5HT or PGE_1. Without this correction, measured removal would be reduced because isotope counts in blood would reflect both unchanged ^{3}H-PGE_1 or ^{14}C-5HT as well as their respective labeled metabolites, and this might explain the low values of percentage removal of the radiolabeled metabolites (Hammond et al 1977). However, at least for serotonin, Rickaby et al (1981) showed that less than two percent of an injected bolus of ^{3}H-5HT in the isolated blood-perfused dog lung lobe appeared in the venous effluent over the sampling interval in the form of 5 hydroxyindoleacetic acid (5 HIAA) (the metabolite of serotonin). They therefore, concluded that the blood levels of ^{3}H-5HIAA produced in the lungs were small enough to be ignored during the passage of the bolus through the lungs. From data available in the literature (Dobuler et al 1982, Klein et al 1978, Patel and Block 1986) changes in endothelial cell function as assessed by serotonin and prostaglandin appear more likely to be related to a subacute type of damage. Therefore, a late development of injury and the occurrence of PMN sequestration in the lung (or any other associated event) may be required to produce a predominant endothelial cell metabolic dysfunction.

In summary, this study has demonstrated that high concentrations of NO_2 alter the permeability of the alveolar epithelial surface. Furthermore, the observations in the study support the concept that this NO_2-induced injury to the lower respiratory tract of dogs (at least an injury manifesting itself by a "leak" of albumin to the epithelial surface), occurs in the absence of a significant decrease in the removal of trace amounts of ^{3}H-PGE_1 or ^{14}C-5HT . Therefore, it may well be true that inhalation injury is associated with mainly epithelial injury. Thus the rapid changes in alveolar-capillary membrane permeability are not necessarily associated with a demonstrable alteration of an index of endothelial function. A different time course for the expression of these two phenomena can be considered. Depressed metabolic substrate removal appears more likely to be the result of a rather progressive and long-lasting type of injury. Therefore, these results suggest that a true dissociation exists between epithelial permeability properties and metabolic function of pulmonary endothelial cells.

11. CONCLUSIONS

1. The radioaerosol study has conclusively shown that NO_2 altered alveolar epithelial clearance.

2. Altered lung epithelial permeability by radioaerosol scan and BAL Albumin concentration occurred within 1 hour post- NO_2 exposure; recovery occurred within 2 weeks.

3. No decrease in removal of trace amounts of ^{3}H-PGE_1 or ^{14}C-5HT occurred despite epithelial changes.

4. NO_2 inhalation acutely increases the number and type of lung inflammatory cells.

5. Morphological data supported at least a loss of barrier function of the pulmonary epithelial lining.

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